FACULTAD DE CIENCIAS
Departamento de Biología Molecular

p38α MAPK in inflammation-associated colorectal cancer

Doctoral Thesis

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Madrid, 2013

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Doctoral thesis submitted to the Autonomous University of Madrid for the degree of Doctor of Philosophy by

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This thesis work was carried out at the ***Spanish National Cancer Research Centre (CNIO)*** *in Madrid and at the* ***Institute for Research in Biomedicine (IRB****) in Barcelona under the supervision of* ***Dr. Ángel Rodríguez Nebreda****.*

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Abbreviations

4-OHT	4-hydroxy tamoxifen
a.u.	Arbitrary units
ACF	Aberrant crypt foci
AOM	Azoxymethane
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
ASK1	Apoptosis signal-regulating kinase 1
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAC	Colitis-associated colorectal tumorigenesis
CD	Crohn's disease
cDNA	Complementary DNA
CIN	Chromosomal instability
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
DAB	3,3-di-amino-benzidine
DAPI	4',6-diamidino-2-phenylindole
DMBA	7,12-dimethylbenzanthracen
DMEM	Dulbecco's Modified Eagle Medium
dNTPs	Deoxyribonucleotides triphosphate
DSS	Dextran sodium sulfate
DTT	DL-dithiothreitol or Cleland's reagent
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EMT	Epithelial-mesenchymal transition
ERKs	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum

FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Hematoxylin & Eosin
HIF-1α	Hypoxia-inducible factor 1α
HNPCC	Hereditary nonpolyposis colorectal cancer
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IHC	Immunohistochemistry
iNOS	Inducible nitric oxide synthase
JNK	Jun N-terminal kinase
KO	Knockout
LPS	Lipopolysaccharide
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MK-2	Mitogen-activated protein kinase-activated protein kinase -2
MMP	Metalloproteinase
mRNA	Messenger RNA
MSI	Microsatellite instability
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAS	Periodic acid-Schiff
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
PRAK	p38-regulated/activated protein kinase
qRT-PCR	Quantitative real time PCR
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate

SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
TEMED	N,N,N′,N′-tetramethylethylenediamine
TGFβ	Transforming growth factor β
TNF-α	Tumor necrosis factor-α
TPA	Tetradecanoyl phorbol 12-acetate
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
WT	Wild type

Abstract

p38α is a mitogen-activated protein kinase (MAPK) that is very important for the cellular responses to stress, but also has crucial roles in inflammation and tissue homeostasis. Genetic inactivation of p38α in myeloid cells has provided evidence for the importance of this signaling pathway in cytokine production and inflammatory responses *in vivo*. Recent studies have shown that p38α can suppress initiation of lung and liver tumors *in vivo*. However, there is little evidence for p38α inactivating mutations in tumors, suggesting that cancer cells might use this signaling pathway to control multiple cellular processes. In line with this idea, the use of chemical inhibitors has provided evidence that p38α might positively regulate proliferation in some human cancer cell lines.

Given the role of p38α in orchestrating inflammatory responses while negatively regulating epithelial cell transformation, we have investigated how these two functions are balanced during colitis-associated colorectal tumorigenesis (CAC). We found that downregulation of p38α in intestinal epithelial cells (IECs) increases colitis-associated tumorigenesis in mice without affecting tumor size, suggesting that epithelial p38α signaling suppresses tumor initiation. Accordingly, downregulation of p38α in IEC results in enhanced colitis-associated epithelial damage and inflammation, which may be accounted for by increased paracellular permeability and altered colonic homeostasis observed in p38α deficient IEC. However, we found no differences in colitis-associated epithelial damage, inflammation or tumorigenesis when p38α was specifically downregulated in myeloid cells.

Interestingly, p38α downregulation in the tumor epithelial cells impairs proliferation and enhances apoptosis reducing tumor burden. Our results indicate that p38α signaling plays a dual role in IECs during tumorigenesis, suppressing the initial stages that lead to cell transformation but contributing to colorectal tumor maintenance. Moreover, pharmacological inhibition of p38α reduced colon tumor burden suggesting that inhibitors of this pathway might be therapeutically useful for colon cancer.

Introduction

The mitogen-activated protein kinase (MAPK) family

The cellular responses to different stimuli involve the activation of signaling pathways that integrate the external signals and coordinate the activation of specific intracellular programs. In response to extracellular stimuli, the mitogen-activated protein kinase (MAPK) family regulates many cellular processes such as proliferation, differentiation and survival, as well as specialized cellular functions that depend on the cell type (Roux and Blenis, 2004).

MAPKs are proline-directed Ser/Thr protein kinases that play key role in signal transduction in eukaryotic cells. Activation of MAPKs can be induced by an ample variety of different stimuli such as hormones, growth factors, cytokines and environmental stresses (Kyriakis and Avruch, 2001). In mammals, at least eight different MAPKs have been identified (Bogoyevitch and Court, 2004). These MAPKs include the extracellular signal-regulated kinases (ERKs): ERK1 and ERK2; the Jun-N-terminal kinases (JNKs): JNK1, JNK2 and JNK3; the p38 MAPKs: p38α, p38β, p38γ, p38δ; as well as ERK3, ERK4, ERK5, ERK7 and ERK8 (Sebolt-Leopold and Herrera, 2004; Wagner and Nebreda, 2009). These MAPKs can be classified into conventional (ERKs, JNKs, p38 MAPKs and ERK5) or atypical (ERK3, ERK4, ERK5, ERK7 and ERK8) based on their ability to get phosphorylated and activated by MAP kinase kinases (MAPKKs) **(Figure 1)** (Coulombe and Meloche, 2007). Conventional MAPKs are catalytically inactive without any stimuli and in order to be fully active, require dual Thr and Tyr phosphorylation in their activation loop (Thr-Xaa-Tyr motif). Dual phosphorylation and activation of conventional MAPKs is achieved via a set of evolutionarily conserved signaling cascade involving a MAPKK (or MAP2K) that is responsible for phosphorylation of MAPK, and a MAPKK kinase (MAPKKK or MAP3K) that phosphorylates and activates MAP2K (Roux and Blenis, 2004). There are in total seven MAP2Ks in mammals, which have some specificity towards MAPKs **(Figure 1).** For example, MEK1 and MEK2 activate ERK 1 and ERK2, MEK5 activates ERK5, MKK4 and MKK7 activate JNKs, and MKK3, MKK6 and MKK4 can activate p38 MAPKs. Of note, there are about

twenty MAP3Ks in mammals, with each of them receives and integrates specific upstream signals (Cuevas et al., 2007; Huang et al., 2009). Interestingly, only six of the twenty MAP3Ks regulate the ERK1/2 pathway, twelve MAP3Ks regulate the JNK pathway and ten MAP3Ks regulate the p38 MAPK pathway. Regulation and activation of ERK5 by MAP3Ks is more restricted as only two MAP3Ks can activate ERK5 (Cuevas et al., 2007). The large number of MAP3Ks and their restricted activation pattern in response to specific stimuli probably determines which MAPKs will be activated and consequently what will be the cellular response to each particular stimulus (Ichijo, 1999; Stalheim and Johnson, 2008). Among conventional MAPKs, the ERK1/2 pathway is known to be preferentially activated by mitogens (Cobb et al., 1994), whereas JNK and p38 MAPKs are activated by stress and pro-inflammatory cytokines **(Figure 1)** (Kyriakis and Avruch, 2012).

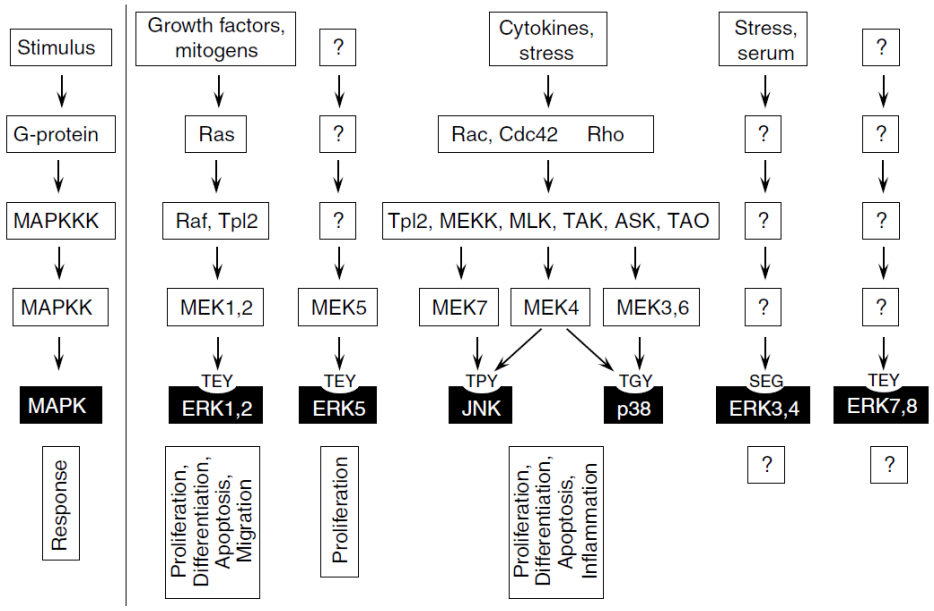


Figure 1. Schematic overview of MAPK pathways.
MAPK family can be divided into two subfamilies, the conventional MAPKs (ERKs, JNKs, p38 MAPKs and ERK5) and the atypical MAPKs (ERK3, ERK4, ERK5, ERK7 and ERK8). See text for details. (taken from (Dhillon et al., 2007))

Once activated, MAPKs phosphorylate downstream targets including transcription factors, protein kinases and many other proteins (Kyriakis and Avruch, 2012; Ono and Han, 2000; Trempolec et al., 2013a). Since a given stimulus can activate different MAPKs as well as different isoforms within each MAPK pathway, it is possible that cross-talk among different MAPKs contributes the final response.

Noteworthy, much less is known about the activation and regulation of atypical MAPKs **(Figure 1)**. No upstream MAP2Ks or MAP3Ks have been identified so far for atypical MAPKs (Bogoyevitch and Court, 2004) suggesting that these MAPKs might not be activated in the conventional and evolutionarily conserved fashion described above.

p38 MAPKs

p38 MAPKs are strongly activated in response to stress stimuli. The family consists of four members: p38α, p38β, p38δ and p38γ, which are approximately 60% identical in their amino acid sequence. The four p38 MAPKs are encoded by different genes and also have different tissue expression patterns. p38α has been considered the dominant isoform within p38 MAPKs based on its significant level of expression in most cell types. p38α is also known as MAPK14 and is the homologue of Hog1 in *Saccharomyces cerevisiae*. Other family members seem to be expressed in a tissue-specific manner; for example, p38β in brain, p38γ in skeletal muscle and p38δ in endocrine glands (Cuadrado and Nebreda, 2010). Of note, p38α knock-out mice are embryonic lethal (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Tamura et al., 2000) while deficiency of p38β, p38δ and p38γ does not affect normal development in mice (Beardmore et al., 2005; Sabio et al., 2005). Functional redundancy has been demonstrated in the case of p38α and p38β, where specific deletion of both genes in the epiblast derivatives of both genes results in additional phenotypes compared with the single knockouts (del Barco Barrantes et al., 2011).

Upstream activators of p38 MAPKs

Like other MAPKs, p38 family members are activated by dual phosphorylation catalyzed by MAP2Ks. In response to appropriate signals, threonine and tyrosine residues in the activation loop (Thr-Gly-Tyr) of p38 MAPKs can be phosphorylated by three different MAP2Ks **(Figure 2)**. MKK6 is a potent activator of all four p38 MAPK family members, whereas MKK3 is unable to activate p38β (Cuadrado and Nebreda, 2010; Enslen et al., 1998; Zarubin and Han, 2005). It is noteworthy that MKK6 and MKK3 share 80% amino acid sequence homology (Stein et al., 1996) and both are highly selective for p38 MAPKs but do not activate JNKs and ERK1/2 (Cuenda and Rousseau, 2007). In addition to MKK3 and MKK6, MKK4 (an upstream activator of the JNKs) can also activate p38α (Brancho et al., 2003; Doza et al., 1995). The relative contribution of each MAP2K to p38α activation is stimulus and cell type dependent as MAP2Ks expression levels vary among cell types (Brancho et al., 2003; Otto et al., 2012). For example, MKK3 has been shown to be the main p38α activator in transforming growth factor b1 (TGF-β1) stimulated mesangial cells (Wang et al., 2002a) whereas MKK6 seems to play a major role in thymocytes (Tanaka et al., 2002) .

In addition to the canonical activation of p38α by upstream kinases, MAP2K-independent alternative mechanisms have been reported. For example, in some cases p38α seems to be activated by autophosphorylation after interaction with TAB1 (transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein) (Ge et al., 2002). An additional alternative mechanism of p38α activation in T-lymphocytes involves phosphorylation of p38α on Tyr 323 by T cell receptor-proximal tyrosine kinases ZAP70 and $p56^{lck}$, which leads to p38α autophosphorylation in its activation loop (Salvador et al., 2005).

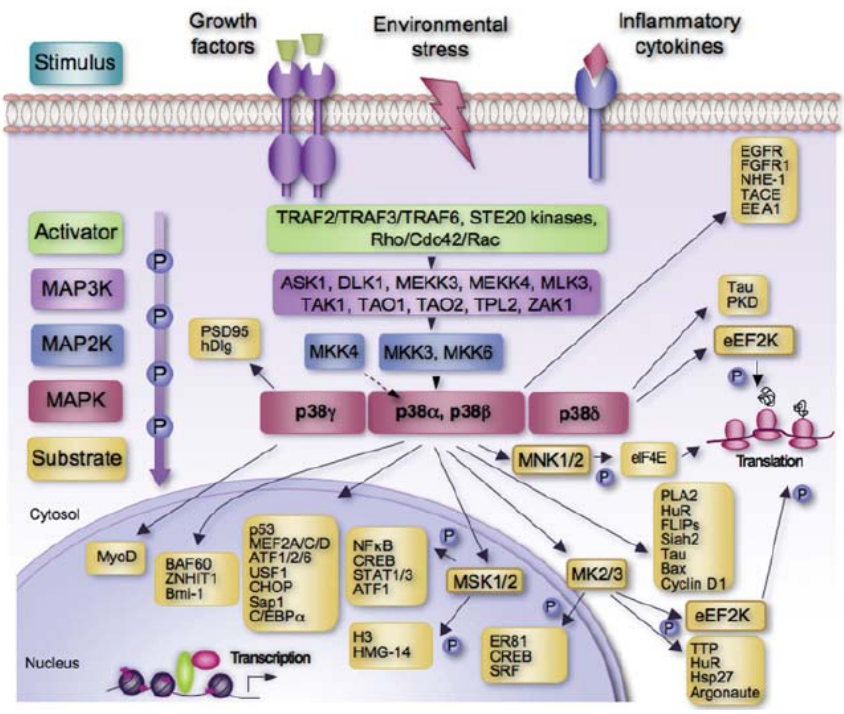


Figure 2. The p38 MAPK pathway.
Different stimuli such as growth factors, inflammatory signals or environmental stresses result in the activation of MAPKKKs. MAPKKKs activate MAPKKs, which in turn activate downstream p38 MAPKs. Activated p38 MAPKs then phosphorylate several downstream targets including protein kinases and transcription factors. (taken from (Cuadrado and Nebreda, 2010))

MAP2Ks are in turn activated by phosphorylation by a MAP3Ks. In mammals, twenty MAP3Ks have been identified so far. About ten MAP3Ks have been shown to elicit p38 MAPK activation (Cuadrado and Nebreda, 2010; Stalheim and Johnson, 2008). MAP3Ks that can activate p38 MAPKs include TAK1 (TGF β-activated kinase 1), ASK1 (apoptosis signal-regulating kinase 1), DLK1 (dual-leucine-zipper-bearing kinase 1), TAO (thousand-and-one amino acid) 1 and 2, TPL2 (tumor progression loci 2), MLK3 (mixed-lineage kinase 3), MEKK (MAPK/ERK kinase kinase) 3 and 4, and ZAK1 (leucine zipper and sterile-a motif kinase 1) **(Figure2)**. Interestingly, some MAP3Ks have been shown to be preferentially activated by specific stimuli, thus integrating particular signals with

specific MAPK pathways. For example, TAK1 has been shown to be preferentially activated by pro-inflammatory cytokines (Stalheim and Johnson, 2008), whereas ASK1 plays a role in the activation of p38α by oxidative stress (Dolado et al., 2007). Some of the MAP3Ks that activate p38 MAPKs can also activate other MAPKs. The diversity of MAP3Ks and their ability to integrate many different stimuli to p38 MAPKs and other signaling pathways provides great versatility to this signaling pathway (Cuevas et al., 2007; Kyriakis and Avruch, 2012).

Downstream targets of p38 MAPKs

Once activated, p38 MAPKs can regulate and phosphorylate many substrates ranging from protein kinases to transcription factors. Many p38 MAPK substrates have been identified based on the use of pyridinyl imidazole inhibitors, such as SB203580 and SB202190, which specifically inhibit p38α and p38β at low concentrations (Eyers et al., 1998; Goedert et al., 1997). Moreover substrate specificity has been sometimes confirmed using cells derived from mice with targeted deletion of each p38 MAPKs (Beardmore et al., 2005; Heinrichsdorff et al., 2008; Sabio et al., 2005).

Protein kinases

Several protein kinases have been identified to be phosphorylated by p38 MAPKs such as MAPK-activated protein kinase 2 (MAPKAPK2 or MK2), MK3, MK5, MAPK-integrating kinase (MNK) 1 and 2, mitogen and stress-activated protein kinase (MSK) 1 and 2 (Cuadrado and Nebreda, 2010; Kyriakis and Avruch, 2012; Zarubin and Han, 2005). Once activated, these protein kinases can phosphorylate various proteins involved in transcriptional regulation (Perdiguero and Muñoz-Cánoves, 2008; Roux and Blenis, 2004) **(Figure 3)**. For example, MSK1 and MSK2 can directly phosphorylate and activate transcription factors such as ATF1, CREB, NFkB isoform p65 and STAT (signal transducer and activator of transcription) 1 and 3 (Arthur, 2008). MK2 and MK3 can control gene expression mainly at post-transcriptional level by phosphorylating tristetraprolin (TTP) and HuR, which in

turn regulate mRNA stability (Kotlyarov and Gaestel, 2008; Rodriguez-Gabriel and Russell, 2008). Lastly, MNK1 and MNK2 can phosphorylate the initiation factor elF4E (eukaryotic initiation factor 4E) to regulate protein synthesis (Mahalingam and Cooper, 2001; Waskiewicz et al., 1999). Interestingly, among these protein kinases MK2/3 are preferentially activated by p38 MAPKs whereas MSKs and MNKs can be phosphorylated and activated by both p38 MAPKs and ERKs. Similarly, MK5 can be also activated by atypical MAPKs ERK3 and ERK4 (Kotlyarov and Gaestel, 2008).

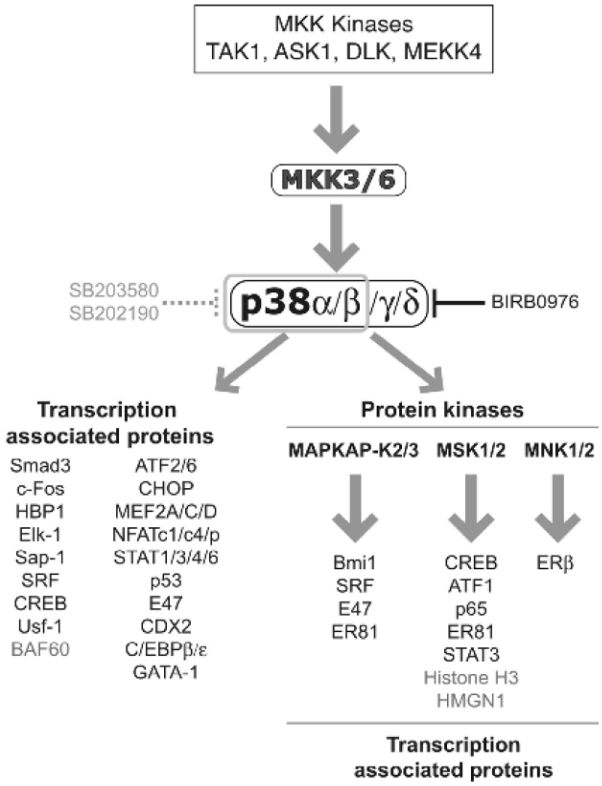


Figure 3. Downstream targets of p38 MAPKs.
After activation by upstream kinases, p38 MAPKs phosphorylate Ser/Thr residues of their substrates, which include transcription-associated proteins and protein kinases. In turn, protein kinases phosphorylate additional transcription-associated proteins to regulate gene expression. (taken from (Perdiguero and Muñoz-Cánoves, 2008))

Transcription factors

A major group of substrates that are phosphorylated by p38 MAPKs include transcription factors, such as ATF (activating transcription factor) 1, 2 and 6, p53, C/EBPβ, myocyte enhance factor MEF2A, MEF2C, NFAT, c-FOS, CREB and SRF accessory protein (Sap1) (Perdiguero and Muñoz-Cánoves, 2008). These transcriptional factors regulate gene expression in a variety of cell processes. For example, p53 induces expression of proteins involved in cell cycle arrest (i.e. p21Cip1, 14-3-3) and apoptosis (i.e. Apaf-1, Noxa) (Bensaad and Vousden, 2005). MEF2 transcription factor regulate the expression of muscle-specific genes which are required for muscle differentiation (Lluis et al., 2006), and NFkB induces expression of many pro-inflammatory and pro-survival proteins (Karin, 2006). Additionally, p38 MAPK signaling can sometimes repress gene transcription. For example, p38α can phosphorylate the transcriptional repressor HBP1 (HMG-box protein), which in turn block the expression of various genes including cyclin D1 and c-myc (Yee et al., 2004). As mentioned above, other transcriptional factors can be regulated indirectly through downstream kinases **(Figure 3)**. In addition to direct or indirect regulation of transcription factors, p38 MAPKs can also regulate gene expression in a transcription factor-independent manner. For example, phosphorylation and activation of TATA-binding protein (TBP) by p38 MAPK at NFκB and AP-1 dependent promoters induces higher affinity TBP-TATA box interaction, which in turn enhances the frequency of transcriptional initiation (Carter et al., 1999; Carter et al., 2001; Crump et al., 2008).

In total, about 96 proteins including not only transcription factors and protein kinases but also DNA- and RNA-binding proteins as well as membrane, mitochondrial and structural proteins have been described to be directly phosphorylated by p38α (Trempolec et al., 2013b). Given the number and type of substrates that can be regulated by p38α, it is not surprising that the p38 MAPK pathway has a key role not only in the stress response but also in many other

functions such as proliferation, apoptosis and differentiation. In normal conditions, MAPKs including p38α should be tightly regulated. Mis-regulation of p38α signaling can lead to diseases such as cancer.

Cancer

Cancer is a complex disease that arises through a multistep, mutagenic process. During malignant transformation, normal cells acquire some key features such as unlimited proliferation, self-sufficiency in growth signals, insensitivity to antigrowth signals and evasion of apoptosis **(Figure 4)**. Furthermore, tumors evolve to gather support from surrounding stromal cells and to attract new blood vessels to bring nutrients and oxygen (Hanahan and Weinberg, 2000). Many of these features can be brought about by genetic alterations in key oncogenes (gain-of-function mutations, amplification, and/or overexpression) and tumor suppressors (loss-of-function mutations, deletion, and/or epigenetic silencing) (Hahn and Weinberg, 2002).

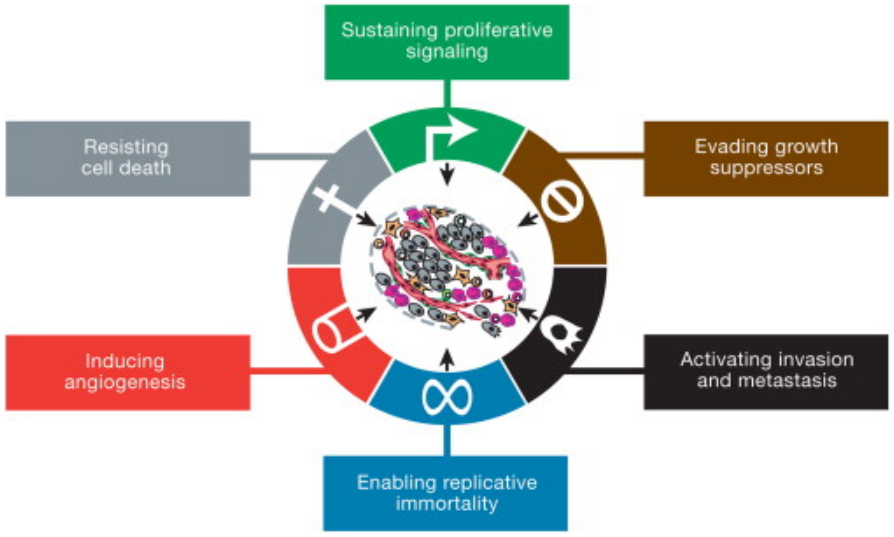


Figure 4. Acquired features during transformation of normal cells to malignant transformation.
(taken from (Hanahan and Weinberg, 2011))

To acquire these features, cancer cells force rewiring and reactivation of existing cellular pathways that are tightly regulated in normal circumstances to control processes such as proliferation, survival and differentiation and to maintain tissue homeostasis (Luo et al., 2009). As mentioned above, p38α MAPK has many potential substrates and can regulate diverse cellular processes such as proliferation, survival, differentiation and inflammation. Accordingly, this signaling pathway has been shown to be important in many pathological conditions including cancer (Coulthard et al., 2009; Wagner and Nebreda, 2009).

p38 MAPKs in cancer

p38α MAPK was originally identified as a protein kinase involved in inflammatory and stress responses (Freshney et al., 1994; Lee et al., 1994; Rouse et al., 1994). Since then p38α has emerged as a key signaling pathway involved in many inflammatory diseases (asthma, rheumatoid arthritis, inflammatory bowel diseases and brain inflammation), cancer and others (Coulthard et al., 2009; Cuenda and Rousseau, 2007; Kumar et al., 2003; Yong et al., 2009).

In recent years, extensive work has been done to expose the roles of p38α signaling in cancer. Although the majority of research on p38α suggests tumor suppressive functions of this pathway, there are also some reports suggesting potential pro-oncogenic functions for p38α. Some of the *in vivo* phenotypes of p38 MAPK signaling pathway related to inflammatory responses and cancer are summarized in **Table 1**, which illustrates a dual role of p38α signaling in cancer.

Mouse line	Phenotype	Model	References
p38α (F/F) × RERTn-Cre	Lung hyperplasia	Lung cancer increased (Kras G12V)	Ventura et al., 2007
p38α (F/F) × Alb-Cre; Mx-Cre	Erythroid proliferation defect	Liver cancer increased (DEN & Pb)	Hui et al., 2007
p38α (F/F) × Alb-Cre; Mx-Cre	Increased ROS & hepatocyte proliferation	Liver cancer increased (DEN)	Sakurai et al., 2008
p38α (F/F) × LysM-Cre	Reduced cytokine production	Sepsis (LPS)	Kang et al., 2008
p38α (F/F) × LysM-Cre; K14-Cre	Reduced inflammatory response	Skin injuy (SDS & UVB)	Kim et al., 2008
p38α (F/F) × LysM-Cre; TieERT2-Cre	No phenotype	ApoE-/- model of Atherosclerosis	Kardakaris et al., 2011
p38α (F/F) × LysM-Cre	Enhanced macrophage apoptosis & plaque necrosis	ApoE-/- model of Atherosclerosis	Seimon et al., 2009
MK-2 -/-	Reduced cytokine production	Sepsis (LPS)	Kotlyarov et al., 1999
MK-2 -/-	Reduced cytokine production	Collagen-induced arthritis	Hegen et al., 2006
MK-2 -/-	Reduced severity to atherosclerosis	Ldlr-/- model of Atherosclerosis	Jagavelu et al., 2007
MK-5 -/- (PRAK)	Impaired ras-induced scenescence	Skin cancer increased (DMBA)	Sun et al., 2007
MK-5 -/- (PRAK)	Impaired tumor growth & angiogenesis	Skin cancer progression reduced (DMBA/TPA)	Yoshizuka et al., 2012
ASK1 -/-	Enhanced inflammatory response	Colorectal cancer increased (AOM/DSS)	Hayakawa et al., 2010
ASK1 -/-; ASK2 -/-	Dual function; ASK1 alone- tumor promoting role ASK1 in coopration with ASK2- tumor suppressive role	Skin cancer (DMBA/TPA)	Iriyama et al., 2009
p38α DN (transgenic)	Reduced edema & inflammation	Skin cancer reduced (solar UV)	Liu et al., 2013
p38α DN (transgenic)	Reduced COX-2 expression	Skin cancer reduced (UVB)	Dickinson et al., 2011
p38α (F/F) × LysM-Cre	Reduced colitis	Colitis model (DSS)	Otsuka et al., 2010
p38α (F/F) × Villin-Cre	Enhanced colitis		

Table 1. ***In vivo*** **roles of the p38α signaling pathway in mouse models of inflammatory diseases and cancer.**

Targeted cells/tissue by the Cre lines: RERTn-Cre (variety of cells/tissues); Alb-Cre (hepatocytes); Mx-Cre (liver and lymphocytes); LysM-Cre (myeloid cells); K14-Cre (ectoderm and its derivatives); TieER^{T2}-Cre (endothelial cells); Villin-Cre (Intestinal epithelial cells).

MK-2 and MK-5 are downstream targets of p38α; ASK-1 and ASK-2 are MAP3Ks that can activate both JNK and p38 MAPKs.

Tumor suppressing roles of p38α signaling

Proliferation

The first evidence in support of a tumor suppressive function for p38α was based on the observation that p38α signaling negatively regulates oncogenic Ras-induced cell proliferation (Chen et al., 2000; Wang et al., 2002b). Accordingly p38α can negatively regulate cell cycle progression both at the G1/S and the G2/M phases of the cell cycle by several mechanisms (Bulavin and Fornace, 2004). For example, p38α has been shown to induce G1/S cell cycle arrest in response to stimuli such as osmotic stress, reactive oxygen species (ROS) and inducers of cellular senescence (Thornton and Rincon, 2009). p38α can activate the G1/S checkpoint through the tumor suppressor p53 and subsequent transcriptional activation of the cell cycle inhibitor p21 (Kishi et al., 2001), by HuR dependent stabilization of p21 mRNA (Lafarga et al., 2009) or by negatively regulating the expression of cyclin D1 directly or through stabilization of cyclin D1 transcriptional repressor, HBP1 (Casanovas et al., 2000; Lavoie et al., 1996; Yee et al., 2004). Moreover, p38α can induce G1/S checkpoint by other mechanisms (Thornton and Rincon, 2009).

Apart from the G1/S checkpoint, p38α has been also associated with the G2/M phase of cell cycle (Bulavin et al., 2002; Diehl et al., 2000; Garner et al., 2002; Mikhailov et al., 2005). The p38α substrate MK-2 can mediate G2/M arrest by phosphorylation of Cdc25B and Cdc25C in response to UV irradiation (Manke et al., 2005). Independently of the stress response, p38α has been also shown to negatively regulate proliferation of various types of primary cells, including cardiomyocytes, hepatocytes, fibroblasts, haematopoietic cells and lung cells (Wagner and Nebreda, 2009).

Apoptosis

Another tumor suppressive function of p38α involves apoptosis induction. Activation of p38α has been shown to mediate apoptosis in various types of cells such as cardiomyocytes, thymocytes, keratinocytes and melanocytes (Chen et al.,

2005; Kim et al., 2003; Purcell et al., 2001; Van Laethem et al., 2004) following different stimuli including chemotherapeutic drugs, UV irradiation, oxidative stress and serum starvation (Brozovic and Osmak, 2007; Bulavin et al., 1999; Dolado et al., 2007; Fassetta et al., 2006; Olson and Hallahan, 2004; Porras et al., 2004). p38α induces apoptosis by both transcriptional and post-translational mechanisms, which affect death receptors, survival pathways or Bcl-2 family proteins. The mechanisms through which p38α induces apoptosis seem to be stimulus and cell type dependent (Dolado and Nebreda, 2008).

Differentiation

In addition to the anti-proliferative and apoptotic activities, p38α can also regulate differentiation of many cell types (Cuenda and Rousseau, 2007; Oeztuerk-Winder and Ventura, 2012). In several cancer cell lines activation of p38α seems to elicit more differentiated and less transformed phenotypes (Finn et al., 2004; Puri et al., 2000). Mechanistically, p38α can directly phosphorylate several transcriptional factors involved in tissue specific differentiation (Cuenda and Rousseau, 2007; Wagner and Nebreda, 2009). *In vivo* studies in mice have also revealed the importance of p38α in myoblast differentiation (Perdiguero et al., 2007), intestinal epithelial cell differentiation (Otsuka et al., 2010) and in adult cardiomyocytes (Engel et al., 2005).

The importance of the differentiation inducing activity of p38α in tumor suppression was illustrated in recent reports using mouse models. Thus, p38α-deficient mice are more susceptible to K-ras induced lung tumorigenesis, and hepatocyte-specific deletion of p38α promotes DEN/Pb-induced liver cancer. In the case of K-ras induced lung tumorigenesis, the tumor suppressor activity of p38α was attributed to deregulated proliferation and impaired differentiation of the lung stem and progenitor cells (Ventura et al., 2007) while in the case of hepatocellular cancer deficiency of p38α induces hyperproliferation of hepatocytes

and liver cancer cells (Hui et al., 2007). In line with these results, reduced expression of the p38α protein or reduced phosphorylation of p38 MAPK was found in human lung tumors and hepatocellular carcinoma compared with normal tissues (Iyoda et al., 2003; Ventura et al., 2007). Altogether, these observations strongly suggest that p38α functions as a tumor suppressor.

Tumor promoting roles of p38α signaling

Proliferation/ apoptosis

In contrast to the tumor suppressor function of p38α, several reports indicate pro-survival roles of p38α. As described above, p38α negatively regulates proliferation in some cell lines but there are also reports indicating that p38α can sometimes positively regulate proliferation in hematopoietic cells and in several human cancer cell lines (Comes et al., 2007; Halawani et al., 2004; Neve et al., 2002; Platanias, 2003; Recio and Merlino, 2002; Ricote et al., 2006; Wang et al., 2010). One of the early example came by the observation that proliferation of PC12 and Swiss 3T3 cells, induced by the growth factor FGF-2, requires p38 MAPK activation as it is blocked by the chemical inhibitor SB203580 (Maher, 1999).

p38α has also been implicated in establishing the G2/M checkpoint in response to various cytotoxic agents such as etoposide and doxorubicin. Inhibition of p38α after treatment with these cytotoxic agents increases apoptosis (Cappellini et al., 2005; Kurosu et al., 2005; Reinhardt et al., 2007). The survival effect of p38 MAPK in response to DNA damage could be explained by its role inducing cell cycle arrest and facilitating DNA repair prior to mitosis entry, helping tumor cells to escape mitotic catastrophe. In non-small cell lung cancer cells, p38α mediates cell survival after γ-irradiation by increasing the expression and activity of DNA-repair proteins (Cosaceanu et al., 2007). In some cases p38α plays a prosurvival role in G2 arrested cells through the coordinated downregulation of proapoptotic genes and upregulation of prosurvival genes (Phong et al., 2010). In the case of brain and

thymocytes, it has been shown that p38α can phosphorylate and inactivate GSK3β and as a consequence β-catenin is accumulated and provides survival signals (Thornton et al., 2008). However, implication of this mechanism in cancer has not been reported.

Inflammation

p38α was discovered as a kinase that regulates the inflammatory response. The importance of inflammation in cancer initiation and progression has been extensively reviewed (Balkwill and Mantovani, 2001; Hussain and Harris, 2007; Mantovani et al., 2008). p38α plays an important role in the production of inflammatory mediators and several pro-inflammatory cytokines. For example, p38α regulates the induction of the pro-inflammatory protein cyclooxygenase 2 (COX2) (Gauthier et al., 2005). COX-2 is overexpressed in several malignancies (Subbaramaiah and Dannenberg, 2003) and has been correlated with bad prognosis and poor survival in breast and colon cancer (Hoellen et al., 2011; Wang and Dubois, 2010). Furthermore, p38α regulates the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 (Kumar et al., 2003; Wagner and Nebreda, 2009). p38α can also control cytokine expression by regulating NFkB (Saccani et al., 2002). Genetic inactivation of p38α in myeloid cells has also provided evidence of p38α importance in cytokine production and inflammatory responses *in vivo*, including models of LPS-induced sepsis, SDS and UVB-induced skin injury and DSS-induced colitis (Kang et al., 2008; Kim et al., 2008a; Otsuka et al., 2010).

Invasion and angiogenesis

Invasion and angiogenesis are two processes that play important roles in tumor progression and metastasis. p38α regulates these two processes and thus can have a direct role in tumor progression (del Barco Barrantes and Nebreda, 2012). For example, p38α can enhance Twist1 protein stability by phosphorylation, which in turn induces epithelial-mesenchymal transition (EMT) and invasiveness of

mammary epithelial cells (Hong et al., 2011). Furthermore, p38α can also regulate the expression of matrix metalloproteinases (MMPs) such as MMP-1, MMP-2, MMP-9 and MMP-13 in several human cancer cell lines (Hsieh et al., 2010; Johansson et al., 2000; Kumar et al., 2010; Park et al., 2011; Xu et al., 2006). MMPs play key role in extracellular matrix remodeling and degradation by metastatic cells (Coussens et al., 2002). Inhibition of p38α in cancer cell lines results in reduced cell invasion. Angiogenesis is also very important for tumor progression and metastasis. Vascular endothelial growth factor (VEGF), an inducer of tumor survival and angiogenesis (Carmeliet, 2005), has been shown to be regulated by p38α (Yoshino et al., 2006). Moreover, p38α can activate hypoxia-inducible factor 1α (HIF-1α) through the stabilization of its α-subunit (Emerling et al., 2005). HIF-1α is a transcription factor that regulates the expression of angiogenic growth factors and cytokines such as VEGF and TGFβ.

A recent report has also suggested an *in vivo* role of p38α signaling in tumor promotion (Yoshizuka et al., 2012). This study shows that p38-regulated/activated protein kinase (PRAK) is activated in host endothelial cells and mediates tumor angiogenesis for sustained growth and malignant progression of the skin tumors induced by 7,12-dimethylbenzanthracene (DMBA)/ tetradecanoyl phorbol 12-acetate (TPA). Of note, PRAK deficiency enhances one stage skin carcinogenesis induced by DMBA by preventing senescence and thus promoting oncogenic transformation (Sun et al., 2007). Another recent paper has also described a pro-tumorigenic role of p38α by using p38α dominant negative (p38α-DN)-expressing transgenic mice in UVB-induced skin carcinogenesis, expression of p38α-DN results in decreased epidermal proliferation, in part through inhibition of AP-1 activity, and a reduction in COX-2 protein levels (Dickinson et al., 2011).
Additionally, increased phosphorylation of p38α has been correlated with several human malignancies such as follicular lymphoma (Elenitoba-Johnson et al., 2003), non-small cell lung cancer (Greenberg et al., 2002), breast carcinomas (Esteva et al.,

2004), gliomas (Demuth et al., 2007), head and neck squamous cell carcinomas (Junttila et al., 2007) and hepatocellular carcinomas (Wang et al., 2012).

All this evidence suggests that p38α signaling might have a dual role in tumor initiation and progression. From the tumorigenesis experiments in mice, it seems that p38α has a tumor suppressive function during tumor initiation while its tumor promoting function could be mainly confined to tumor progression. Noteworthy, in tumor initiation and progression many signaling pathways including p38 MAPKs are involved, which may be regulated in a different way depending on the cell type, stimuli and stage of tumorigenesis. Thus, further work including the use of specific mouse models to modulate the p38α signaling pathway in a time and tissue specific manner should help to define better the roles of p38α in tumor initiation and progression.

Colorectal Cancer

More than 1 million new cases of colorectal cancer (CRC) are diagnosed worldwide each year (Tenesa and Dunlop, 2009). Colorectal cancer is the third most common form of cancer and the fourth common cause of death from cancer worldwide. The frequency of CRC shows a considerable geographical variation with highest incidences in developed countries, lowest in Africa and South-Central Asia, and intermediate in Latin America (IARC 2008). Many agents contribute to CRC development including diet and lifestyle as well as inherited and somatic mutations. Among the dietary and lifestyle risk factors for CRC are a diet rich in unsaturated fats, a high intake of red meat and a low intake of vegetables, excessive alcohol consumption, cigarette smoking and overweight and lack of exercise (Chao et al., 2000; Huxley et al., 2009; Potter, 1999; Slattery, 2000).

In CRC, carcinogenesis is a multistep process from the normal epithelium to dysplastic precursor lesions and to carcinoma **(Figure 5)**, regardless of the underlying etiology (Fearon and Vogelstein, 1990; Ullman and Itzkowitz, 2011).

CRC can be divided into three main types according to the way that it has developed, which are hereditary, sporadic and inflammatory (Ishikawa and Herschman, 2010).

Figure 5. Multistep genetic model of colorectal tumorigenesis
(taken from (Pino and Chung, 2010))

Hereditary colorectal cancer

This group can be further divided into two major subgroups; hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), based on the presence of polyposis. Both HNPCC and FAP are characterized by an early age (approximately 45 years) of CRC appearance with predominance of right-sided colorectal cancer (Lynch and de la Chapelle, 2003).

Hereditary nonpolyposis colorectal cancer

HNPCC is also known as Lynch syndrome and cancer family syndrome. Among all CRC cases, about 1-6% are related to HNPCC (Lynch and de la Chapelle, 2003). HNPCC is an autosomal dominant condition caused by a germline mutation in DNA mismatch-repair (MMR) genes which results in microsatellite instability (MSI). Furthermore, resulting MSI promotes mutations in other genes that contribute to colon tumorigenesis (Kwak and Chung, 2007). Mutations in beta-catenin, TGFβRII, Bax, APC, p53 and K-ras genes have been also identified in HNPCC tumors, which might be due to MSI (Fearon, 2011; Lynch and de la Chapelle, 2003).

Familial adenomatous polyposis

FAP is a rare autosomal dominant disease with almost 100% penetrance and characterized by presence of hundreds to thousands of colorectal adenomas. FAP accounts for approximately 1% of all CRC (Kwak and Chung, 2007). FAP is caused by mutations in tumor suppressor gene adenomatous polyposis coli (APC) (Groden et al., 1991; Kinzler et al., 1991). However, inactivation of APC also occurs in sporadic colorectal cancer (see below). APC regulates the degradation of β-catenin and inactivation of APC leads to accumulation of β-catenin, which activates the transcription of growth-promoting genes such as cyclin-D1 and c-myc (Chung, 2000).

Sporadic colorectal cancer

Colorectal tumors that are not associated with hereditary cancer syndromes are defined as sporadic colorectal cancer. The lifetime risk of developing sporadic colorectal cancer is approximately 5% (Jemal et al., 2002). The development of CRC typically follows several consecutive steps (Fearon and Vogelstein, 1990). Genomic destabilization is an early step in sporadic tumor development. Loss of APC function is among the earliest molecular events in colorectal cancer, leading to activation of Wnt/β-catenin signaling pathway and subsequent transcription of target genes (Chung, 2000; Morin et al., 1997). Furthermore, mutations in the K-ras oncogene have been found in 15-68% of sporadic colorectal adenomas and in 40-50% of cancers (Takayama et al., 2006). Loss of p53 tumor suppressor function has been also identified in 40-50% of sporadic colorectal cancers which believed to occur at the time of transition from adenoma to carcinoma (Itzkowitz and Yio, 2004). However, the somatic mutation spectrum in the colon cancer seems to be more extensive. Approximately 90 mutant genes were identified in a systematic sequencing analysis of colorectal cancers, of which 69 genes were considered related to the pathogenesis of CRC (Sjoblom et al., 2006). Moreover, epigenetic factors such as promoters of CpG island methylation also contribute to colon

cancer carcinogenesis by silencing promoters of tumor suppressors (Draht et al., 2012; Santini et al., 2001; Toyota et al., 1999).

Inflammation-associated colorectal cancer

Inflammation and cancer

The connection between inflammation and cancer is not new. In 1863, Rudolf Virchow noted that inflammatory cells are present in tumors and proposed that inflammation contribute to the development of cancer (Balkwill and Mantovani, 2001). It is estimated that about 15-20% of all cancer related deaths are linked to infections and inflammation (Mantovani and Pierotti, 2008). Tumor development involves two main steps: initiation and promotion. In the initiation, somatic cells accumulate irreversible DNA sequence alterations (mutation of oncogenes, tumor suppressor genes and other key regulators of cell proliferation). The promotion step is associated with continuous or repeated stimuli which lead to induction of cellular proliferation and changes in the cellular microenvironment that promote tumor formation. It is now widely accepted that chronic inflammation plays an instrumental role in the promotion step of tumor development (Coussens and Werb, 2002; DiDonato et al., 2012).

Recent epidemiological studies have identified inflammation and infections as major risk factors for many kinds of cancer, such as infection of hepatitis B and liver cancer **(Table 2)**. Both exogenous (infections and toxic compounds) and endogenous (inherited diseases and obesity) inducers of inflammation can contribute to chronic inflammation induced cancer (Medzhitov, 2008).

Disease	Type of cancer	Increased risk
Auto-inflammatory/non-infectious		
Crohn's disease	Colon cancer	3
Ulcerative colitis	Colon cancer	6
Chronic pancreatitis	Pancreatic cancer	2–50
Endometriosis	Endometrial cancer	1.4
Hemochromatosis	Liver cancer	219
Thyroiditis	Thyroid cancer	3
α-1-Anti-trypsin deficiency	Liver cancer	20
Acquired		
Viral		
Hepatitis B	Liver cancer	88
Hepatitis C	Liver cancer	30
Epstein–Barr virus	Hodkin's and Burkitt's lymphoma	4
Bacterial		
Helicobacter Pylori	Gastric cancer	11
Pelvic inflammatory disease	Ovarian cancer	3
Chronic protatitis	Prostate cancer	2–3
Parasitic		
Schistosoma hematobium	Bladder cancer	2–14
Schistosoma Japonicum	Colon cancer	2–6
Liver fluke	Cholangiocarcioma and liver cancer	14
Chemical/physical/metabolic		
Alcohol	Multiple cancers (including liver, pancreas, head and neck cancer)	2–7
Asbestos	Mesothelioma	>10
Obesity	Multiple cancers	1.3–6.5
Tobacco smoke and inhalation of other noxious chemicals	Lung cancer (and multiple other cancers)	>10
Gastric reflux, Barrett's esophagus	Esophageal cancer	50–100

Table 2.Chronic inflammation or infection increases cancer risk.
(taken from (Schetter et al., 2010))
Increased risk is expressed by the odds ratio or relative risk.

Inflammatory bowel disease and colorectal cancer

Inflammatory bowel diseases (IBD) including crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic, progressive and relapsing inflammatory

disorders mainly in the large bowel. Genetic, environmental and intestinal microbial factors seem to be important contributors in the etiology and pathogenesis of IBD (Schirbel and Fiocchi, 2010). Existing evidence indicate that IBD patients are associated with higher risk of development of colitis-associated colon cancer (CAC) (Ekbom et al., 1990a; Ekbom et al., 1990b; Gillen et al., 1994). However, UC patients have higher risk for CAC than CD patients (Grivennikov and Karin, 2010; Schetter et al., 2010). The risk of developing CRC is directly related to the duration and extent of colitis. CRC is rarely detected in patients who have had colitis for less than 7 years but afterward the risk increases at a rate of 0.5%-1% per year (Ullman and Itzkowitz, 2011). The extent of colitis (severity of inflammation) is the second important factor related to CAC risk; the more colonic surface that is affected by colitis, the greater the risk of CAC (Rutter et al., 2004).

Compared to sporadic colorectal cancer, CAC has distinctive clinical features. Regardless of the underlying condition, most CRCs develop from a dysplastic precursor lesions (Fearon and Vogelstein, 1990; Ullman and Itzkowitz, 2011). In sporadic CRC, the dysplastic precursor is usually the adenomatous polyp (adenoma). In contrast, CAC typically arises from dysplastic precursor that can be nonpolypoid, flat, localized or multifocal dysplasia. Moreover, CAC affects individuals at a younger age (Xie and Itzkowitz, 2008).

Microscopically, dysplasia in IBD are divided into five categories: negative for dysplasia, indefinite dysplasia (dysplasia not yet detectable), low-grade dysplasia (LGD, nuclei confined to the basal half of the cells), high-grade dysplasia (HGD, nuclei restricted more to apical side), and CAC (Riddell et al., 1983). Finally, the sequence of molecular events that lead from dysplasia to invasive adenocarcinoma is different in CAC from sporadic CRC (see below).

From Inflammatory bowel disease to Colitis-associated colon cancer

CAC develops in chronically inflamed mucosa and are believed to develop in a sequence of no dysplasia-indefinite dysplasia-LGD-HGD-carcinoma. Tumor progression can skip one or more of these steps (Farraye et al., 2010). As in sporadic CRC, CAC also occurs through sequential events such as somatic mutation and their clonal expansion (Itzkowitz and Yio, 2004). Also similar is the frequency of occurrence of two main types of genomic instability that contribute to colon carcinogenesis. It is estimated that chromosomal instability (CIN) and microsatellite instability (MSI) account for 85% and 15% of colon carcinogenesis, respectively (Xie and Itzkowitz, 2008). However, the timing and frequency of molecular alterations is different in CAC when compared to sporadic CRC **(Figure 6)**. For example, APC loss of function is considered to be a very common early event at a frequency of around 85% in sporadic CAC. However APC loss is rare and usually occurs late in the transition to CAC (Aust et al., 2002; Tarmin et al., 1995; Xie and Itzkowitz, 2008). Loss of p53 function is another important step which occurs late during adenoma to carcinoma transition in sporadic CRC, whereas in patients with colitis, p53 mutations and allelic loss are more frequent (50-85%), occur early and can be detected in mucosa that is either non-dysplastic or indefinite for dysplasia (Brentnall et al., 1994; Burmer et al., 1992; Yin et al., 1993). In fact, p53 mutations were detected in inflamed mucosa of more than 50% UC patients who did not have any tumors, suggesting that chronic inflammation might facilitate these mutations (Hussain et al., 2000). Similar to sporadic CRC, epigenetic alterations such as methylation of CpG islands have been detected throughout the mucosa of UC patients (Hartnett and Egan, 2012; Issa et al., 2001).

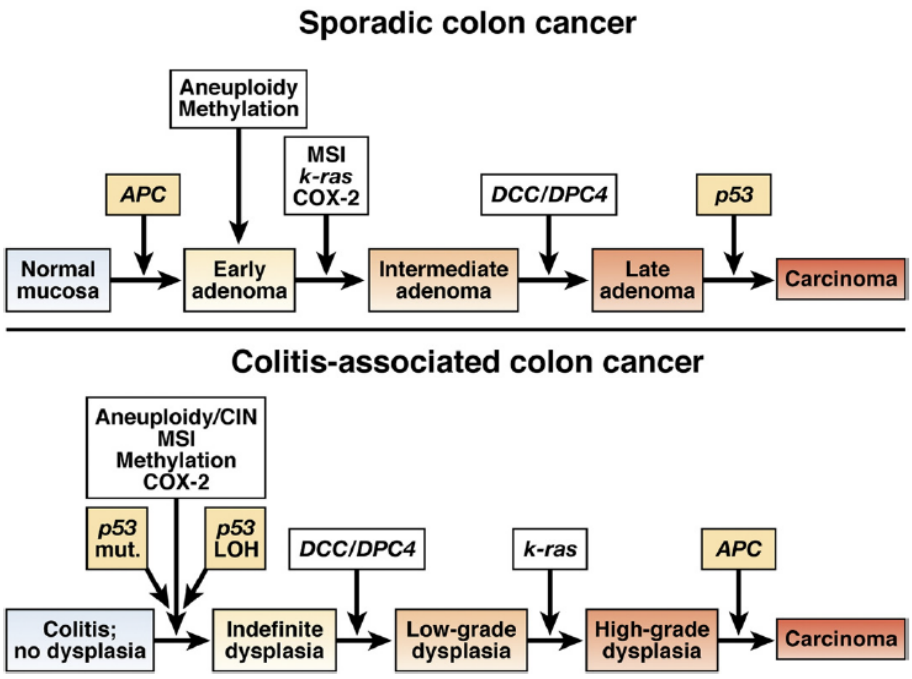


Figure 6. Comparison between colitis-associated colon cancer (CAC) and sporadic colon cancer (CRC).

During the sporadic and colitis-associated colon cancer, there are many similarities including chromosomal instability, DNA methylation, activation of COX2 and oncogene k-ras and mutation and eventual loss of hetrozygosity of p53 and APC. However, the frequency and sequence of these molecular events differ between the two types of colon cancer. (taken from (Ullman and Itzkowitz, 2011))

Mouse model of colitis and colitis-associated cancer

Experimental animal models are essential tools to understand pathogenesis and for preclinical testing of new therapeutic options. To study the hereditary CRC, APC mutant mice was developed and successfully used in many studies (Moser et al., 1990). To study sporadic CRC and CAC experimental models based on chemical carcinogens or pro-inflammatory reagents have been developed. These models are reliable, reproducible and highly efficient in induction of colon tumors in mice (Neufert et al., 2007).

Azoxymethane

Azoxymethane (AOM) and its derivatives have been used successfully in experimental models of CRC. AOM is a chemical agent that can initiate colon cancer by alkylation of DNA (Neufert et al., 2007; Papanikolaou et al., 1998). AOM is a downstream metabolite of dimethylhydrezine (DMH), which was frequently used in the past. In recent years, AOM is widely used because of higher potency and stability in solution (Papanikolaou et al., 1998).

Repeated intraperitoneal injections of AOM induces colon tumor within 30 weeks (Neufert et al., 2007). Tumors induced by AOM resemble human CRC in many ways. AOM-induced tumors are frequently located in the distal part of the colon, start with polypoid growth similar to spontaneous CRC in human. They show similar histopathological features, but AOM-induced tumors often lack mucosal invasiveness and metastasis (Boivin et al., 2003; Nambiar et al., 2003; Shamsuddin, 1984). At the molecular level, AOM-induced tumors are also similar to human CRC. For example, AOM-induced tumors show aberrant expression of APC as well as mutations and altered localization of β-catenin (Maltzman et al., 1997; Takahashi et al., 2000b). Altered expression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) have been also reported (Wang et al., 1998). Moreover, AOM-induced tumors also have mutations in K-ras and elevated levels of COX-2 and inducible nitric oxide synthase (iNOS-2) similar to human CRC (Takahashi et al., 2000a). It has been reported that the genetic background is an important factor in AOM-induced tumorigenesis. Strains of high to moderate susceptibility are A/J, SWR/J or FVB/N (Bissahoyo et al., 2005; Neufert et al., 2007; Papanikolaou et al., 2000). In addition to the genetic background, differences in the intestinal microflora between animal facilities as well as variability of AOM preparations might also affect susceptibility.

As spontaneous colon tumorigenesis induced by AOM alone needs at least 30 weeks, this model is used to study the pathogenesis of sporadic CRC.

Dextran sodium sulfate model of colitis

Dextran sodium sulfate (DSS) is a pro-inflammatory reagent that has been used extensively to induce colitis in experimental models. DSS acts as an irritant to the epithelial lining of colon, resulting in bloody diarrhea (Okayasu et al., 1990; Seril et al., 2003). Colitis induced by DSS has many characteristics similar to human UC, such as signs of diarrhea, shortening of colon, multiple erosions and inflammatory mucosal changes, loss of crypts and ulcerations. Moreover, administration of DSS induces inflammation with high levels of COX-2, iNOS and high intensity staining of β-catenin. To induce chronic inflammation, repeated cycles of DSS can be used. It is widely believed that chronic inflammation is a driving force in the CAC (Clapper et al., 2007).

Typically 1-3 % DSS (wt/vol) in drinking water is given to mice for 3-5 days to induce colitis. Of note, different mouse strains show different susceptibility to DSS (Mahler et al., 1998; Stevceva et al., 1999). Moreover, colon tumorigenesis induced by DSS alone requires a long treatment period with repeated administration of DSS. Additionally, low tumor incidence and multiplicity is also a drawback of this model (Cooper et al., 2000; Okayasu et al., 2002; Tamaru et al., 1993; Yamada et al., 1992).

Azoxymethane/ Dextran sodium sulfate model

To investigate colitis-associated tumor development in more rapid and reliable manner, a two-stage model has been proposed. This includes the combination of a single injection of AOM followed by administration of the pro-inflammatory reagent DSS (Neufert et al., 2007; Okayasu et al., 1996; Tanaka et al., 2003). With this model, different mouse strains develop multiple colon tumors within a period of 20 weeks (Suzuki et al., 2006; Tanaka et al., 2003). Different susceptibility to AOM/DSS is also reported in different mouse strains. For example, incidence of colonic tumors was 100% in Balb/c mice and 50% in C57BL/6N mice; whereas only adenomas developed in 29% C3H/HeN mice (Suzuki et al., 2006). Furthermore,

repeated DSS administration after AOM injection enhances tumor growth resulting in multiple large colon tumors already after 10 weeks (Becker et al., 2005). When comparing this two-stage model of tumorigenesis with studies using AOM alone, DSS displays a strong tumor promoting activity in this model (Suzuki et al., 2004; Tanaka et al., 2003). The flat and polypoid lesions developed by AOM/DSS treatment are positive for nuclear β-catenin. This finding is consistent with the presence of β-catenin mutations in AOM-induced colon tumors and the altered localization of β-catenin in human CAC (Aust et al., 2002; Takahashi et al., 2000b; Tanaka et al., 2005). Moreover, tumors are also positive for COX-2 and iNOS but not for p53 (Tanaka et al., 2003). Further studies showed that blocking tumor necrosis factor alpha (TNF-α) or interleukin-6 (IL-6), two NF-κB regulated cytokines that are important for CAC in human and mice, results in reduced tumor burden and size (Becker et al., 2004; Greten et al., 2004; Grivennikov et al., 2009; Popivanova et al., 2008). These studies support the strong role of inflammation in AOM/DSS model as these two cytokines are mainly produced by inflammatory cells.

Due to the highly reliable and reproducible, cost and time effective experimental protocol that also mimics human IBD and CAC, the AOM/DSS model is one of the most widely used experimental system to investigate CAC in mice.

p38α in inflammatory bowel disease and colorectal cancer

IBD is a disorder that arises from chronic relapsing inflammation. Patients with IBD are associated with higher risk of development of CAC. The duration and extent of colitis (severity of inflammation) are important risk factors to develop colon cancer. Given the role of p38α in inflammatory responses, activation of the p38 MAPK pathway has been evaluated in IBD patients and yielded contradicting results. Some studies report levels of phosphorylated p38 MAPK in IBD patients (Docena et al., 2010; Waetzig et al., 2002), while in other studies p38 MAPK phosphorylation levels were similar in IBD patients compared to controls

(Malamut et al., 2006). Similar contradicting results were obtained from mouse model of colitis. Some groups reported that p38 MAPK inhibition improves the clinical symptoms (Hollenbach et al., 2004), while others reported deteriorating effects (Malamut et al., 2006; ten Hove et al., 2002). In clinical trials, p38 MAPK inhibitors have not shown promising results. In patients with Crohn's disease, the p38 and JNK inhibitor CNI-1493 has shown some clinical improvement (Hommes et al., 2002), while the p38 inhibitor BIRB796 did not show any improvement (Schreiber et al., 2006). Nevertheless, a recent study using specific downregulation of p38α in myeloid cells and intestinal epithelial cells showed opposite function in DSS-model of colitis which could be a possible explanation for the controversial effects reported upon p38 MAPK inhibition. Downregulation of p38α in myeloid cells reduces colitis whereas in IEC promotes colitis. (Otsuka et al., 2010). However, the role of p38α in these studies was not explored in CAC. A study using the p38α/β inhibitor SB2021890 has shown impaired tumor growth in the APC^{min} /AOM model of CRC (Chiacchiera et al., 2009), but the precise contribution of p38α was not characterized.

In this thesis genetic mouse models have been used to inactivate p38α in myeloid cells or IECs combined with the DSS model of colitis or the AOM/DSS model of CAC.

Aim of the work

In recent years, p38α MAPK has been established as a negative regulator of epithelial cell proliferation both in cell cultures and in mouse models, supporting a putative tumor suppressor role. Surprisingly, few reports suggest p38α in some cancer cell lines can positively regulate proliferation and thus argue against the tumor suppressive function of p38α. In addition, p38α has been shown to regulate inflammatory responses by controlling inflammatory mediators. Considering this information, this thesis investigates how p38α regulates colitis-associated colorectal tumorigenesis with the following specific aims:

1. Impact of p38α downregulation in IECs using the AOM/DSS model of colorectal tumorigenesis.

2. Impact of p38α downregulation in epithelial cells of colorectal tumors induced by AOM/DSS.

3. Impact of p38α downregulation in myeloid cells using the AOM/DSS model of colorectal tumorigenesis.

Materials & Methods

Buffers and solutions

Laboratory stock solutions and common buffers were prepared as described in Current Protocols in Cell Biology (2001) under the section Laboratory stock solutions and equipment (web link: http://onlinelibrary.wiley.com/doi/10.1002/0471143030.cba02as00/abstract.)

Tail Buffer for mice genotyping

100 mM NaCl, 50 mM Tris-HCl (pH 8), 10m M EDTA (pH 8), 1% SDS in sterile water

Cell/tissue lysis buffer

1% NP40, 150 mM NaCl, 50 mM Tris HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 20 mM sodium fluoride, 2 mM PMSF, 2 μM microcystin, 2 mM Sodium orthovanadate, 1 mM DTT and 1x EDTA-free complete protease inhibitor cocktail (Roche, #11873580001)

Laemmli-SDS-PAGE gels

Resolving gels,

Volume (for 10ml)	**10%**	**12%**
40% Acrylamyde/Bis (29:1)	2.5 ml	3 ml
1M Tris-HCl pH 8.8	2.5 ml	2.5 ml
10% SDS	100 μl	100 μl
Water	4.792 ml	4.292 ml
10% APS	100 μl	100 μl
TEMED	8 μl	8 μl

Stacking gels (4%),

Volume	**5 ml**	**10 ml**
40% Acrylamyde/Bis (29:1)	0.5 ml	1 ml
1M Tris-HCl pH 6.8	1.25 ml	0.625 ml
10% SDS	100 μl	50 μl

Water	7.54 ml	3.77 ml
10% APS	100 µl	50 µl
TEMED	10 µl	5 µl

Electrophoresis buffer (10X)

30 g of Tris base

10 g of SDS

144 g of Glycine

Dissolved in distilled water and bring volume up to 1 L.

Transfer buffer (10X)

30.3 g of Tris base

1 g of SDS

144 g of Glycine

Dissolved in distilled water and bring volume up to 1 L.

20% methanol was added to 1X transfer buffer before use.

Ponceau Red staining solution *(0.1% w/v) in 5% (v/v) acetic acid*

0.3 g of Panceau Red

10 ml Acetic acid

Dissolved in distilled water, and bring volume up to 200 ml.

Laemmli sample loading buffer (5X)

For 20 ml,

4.5 ml of 1 M Tris pH 6.8

11.5 ml of 87% glycerol

1 g of SDS

1.5 ml of 1% Bromophenol blue

0.8 g of DTT (154.25 $g.mol^{-1}$)

Add up to 20 ml water and rotate on a wheel for 30 min. Store in aliquots at -20ºC.

Antigen retrieval buffers

- Tris-EDTA buffer pH 9 (for 2 L): 2.4 g Tris, 0.74 g EDTA to 2 L water. Adjust pH to 9. Antigen unmasking was done at 97ºC for 20 min.
- Citrate buffer pH 6 (for 200 ml): 0.58 g Sodium citrate in water. Adjust pH to 6 with 1 M citric acid. Antigen unmasking was done at 97ºC for 20 min, except for p38α which was carried at 120ºC for 20 min.
- Proteinase K: Ready to use Proteinase K solution from Dako (#S3020) was used. Antigen unmasking was done at room temperature (RT) for 5 min.

0.1 M Phosphate buffer (pH 7.4) for electron microscopy

3.1 g of $NaH_2PO_4.H_2O$

10.9 g of Na_2HPO_4 (anhydrous)

Dissolved in distilled water and bring volume up to 1 L. The resulting final solution will be of pH 7.4 and can be stored at 4ºC up to 1 month.

Commercial Reagents and Kits

Product	Company	Reference
40% Acrylamide/Bis (29:1)	Bio-Rad	161-0146
APS	Sigma	A3678
Bromophenol blue	Sigma	B8026
BSA	Sigma	A7906
β-mercaptoethanol	Sigma	M7154
Citric acid	Sigma	251275
DAPI (for TUNEL)	Invitrogen	MP-36930
DMEM	Sigma	D5796
DMSO (for cell culture)	Sigma	276855
DNase I	Roche	4716728a
dNTPs mix (10 mM)	Fermentas	R0192
DTT	GE healthcare	17-1318-02

FBS	Thermo Scientific	E6541L
Formalin (10%, buffered)	Sigma	HT501128
Glycerol	Sigma	49782
Glycine	Sigma	G7126
Microcystin-LR	Enzo Life Sciences	ALX350012
Nitrocellulose membrane 0.2 μm	Whatman™	10401396
NP-40	AppliChem	A1694,0250
PBS 10x	Sigma	D1408
Penicillin/Streptomycin (100x)	LabClinics	P11-010
Peroxidase	Dako	S2023
Phenol:chloroform	Sigma	P2069
PMSF	Sigma	P7626
Ponceau red	Sigma	P3504
Primers for PCR and qRT-PCR	Sigma	-
Propidium iodide	Sigma	P4864
RNA mini Kit (Pure Link)	Ambion	12183-018A
SB203580	Axon MedChem	1363
Sodium citrate	Sigma	71497
Sodium dodecyl sulfate	Sigma	71725
Sodium fluoride	Sigma	S7920
Sodium orthovanadate	Sigma	S6508
SuperScript II reverse transcriptase	Invitrogen	18064-022
Sybr Green supermix	Bio-Rad	1708886
Taq polymerase (Biotaq)	Ecogen	21060
TEMED	Sigma	T9281
Triton X-100	Sigma	T9284
TRIZMA-HCl	Sigma	T3253
TRIZMA-base	Sigma	T6066
Trypsin-EDTA	Sigma	T3924

TUNEL	Roche	11684795910
Tween-20	Sigma	P7949

Mice

Mice were housed in the specific pathogen free (SPF) mouse facilities of CNIO (Madrid) and IRB (Barcelona). Mice had free access to regular chow diet and autoclaved sterile water. The animal facility provided a controlled 12 hour light-dark cycle. For breeding, male and female mice were set together at a minimum age of 6 weeks. Litters were weaned at 4 weeks of age and marked with an eartag.

All animal procedures were conducted according to national and EU regulations and protocols were approved by the corresponding Ethics committees.

Generation of conditional mice

p38$\alpha^{lox/lox}$ mice were described previously (Heinrichsdorff et al., 2008; Ventura et al., 2007). p38α-Δ^{IEC} mice and p38α-$\Delta^{IEC\text{-}ERT2}$ were generated by crossing p38$\alpha^{lox/lox}$ mice with Villin-Cre and Villin-CreERT2 mice, respectively (el Marjou et al., 2004; Madison et al., 2002). To activate the tamoxifen-inducible Cre-ERT2 recombinase, mice were injected intraperitoneally for 5 consecutive days with 1 mg/day of 4-hydroxy tamoxifen (4-OHT) (Sigma, H6278) dissolved in corn oil (Sigma, #C8267). Three days after the last 4-OHT injection, p38α downregulation was confirmed by Western blotting. Littermate controls were used in all experiments.

To generate p38α-Δ^{MC} mice, p38$\alpha^{lox/lox}$ mice were crossed with LysM-Cre (Clausen et al., 1999). To check efficiency of p38α deletion, peritoneal macrophages were tested for genomic exon-2 deletion efficiency and western blotting.

Isolation of genomic DNA from mouse tail for genotyping

Mouse tails were digested in 750 µl of Tail buffer with Proteinase K at 56ºC overnight. After overnight digestion, 250 µl of saturated NaCl was added, mixed for 5 min and centrifuged at full speed for 10 min at RT. The supernatant was carefully

taken into a new tube containing 500 µl of isopropanol. Tubes were then kept in an eppendorf mixer for 2 min and centrifuged at full speed for 5 min at RT. The supernatant was discarded carefully without disturbing the DNA pellet. Pellet was washed in 70% ethanol and after drying pellet was resuspended in 100 µl of autoclaved milliQ water. DNA concentrations were measured in a NanoDrop 2000 spectrophotometer.

Mice genotyping was performed by PCR using 25 ng of tail genomic DNA and the following primers and conditions:
Primers for CRE: (amplicon size 520 bp)
Fw: ACGAGTGATGAGGTTCGCAAG
RV: CCCACCGTCAGTACGTGAGAT
Primers for flox-p38α: (amplicon sizes are 121 bp for the WT and 188 bp for the floxed alleles).
Fw: ATGCTACTGTCTGCGCCTCTCT
Rv: CAGCTTCTTAACTGCCACACGA
PCR conditions:
94ºC for 5 min, 94ºC for 30 sec, 55ºC for 45 sec, 72ºC for 30 sec, step 2-4 for 35 cycles, 72ºC for 10 min and 4ºC forever. PCR products were resolved by agarose gel electrophoresis.

Dextran sulfate sodium (DSS)-induced colitis

In order to induce colonic inflammation that resembles UC in human IBD patients, mice were fed for 5 days with 2% DSS (w/v) (MW 36-50 kDa, MP biomedicals, #160110) ad libidum in drinking water. DSS is toxic to the colon epithelium and causes colitis (Okayasu et al., 1990). After DSS treatment, mice were sacrificed at indicated time points and samples were collected for histological and biochemical analysis. During and after the DSS treatment body weight changes were recorded. Body weight loss can be used as an indicator for the severity of DSS-induced colitis.

AOM/DSS-induced colorectal cancer protocol

To induce colon tumors, mice were subjected to a well-established, chemically induced inflammation dependent colon cancer protocol (Neufert et al., 2007; Tanaka et al., 2003). According to this protocol, mice (8-10 weeks old) were injected intraperitoneally with a single dose of AOM (10 mg/kg, Sigma, #A2853) diluted in PBS. After 5 days, 2% DSS was given in the drinking water for 5 days, followed by 14 days of regular drinking water. The DSS treatment was repeated for two additional cycles and mice were sacrificed 100 days after the AOM injection. Colons were removed from animals, flushed with cold PBS, opened longitudinally and fixed as 'Swiss-rolls' in 10% formalin solution (Sigma, #HT-501128) at room temperature overnight before paraffin-embedding. Before fixing the colons, tumor counting and size measurements were performed using a digital caliper in a blinded fashion.

Sacrifice of mice

Mice were euthanized by cervical dislocation.

Analysis of intestinal permeability in mice

In vivo intestinal permeability was determined in 8-10 months old mice by measuring the appearance of FITC-dextran in the blood. Mice were starved overnight and then FITC-dextran (MW 3000-5000; Sigma #FD4) was administered by oral gavage (44 mg/100 g body weight). After 4 h, mice were anesthetized, blood was collected by cardiac puncture and mice were sacrificed. Serum was separated from whole blood using SST tubes (BD #365968). Serum samples were diluted with an equal volume of PBS (pH 7.4) and 100 µl of diluted serum was added to a 96-well microplate. The concentration of FITC in serum was determined by spectrophotofluorometry (BioTek) with an excitation of 485 nm (20 nm band width) and an emission wavelength of 528 nm (20 nm band width) using serially diluted FITC-dextran as standard.

Transmission electron microscopy

Colon pieces were removed from the anesthetized animals and were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (PB) 0.1M pH 7.4 at 4ºC overnight. After four washes with PB, the intestine pieces were postfixed with OsO4 (2 %) for 2 h and washed again with MQ water and PB (10 min each wash). Pieces were dehydrated at 4ºC through a series of acetone concentrations (50, 70, 90, 96, and 100%), prior to being progressively (25, 50, 75 and 100%) embedded in Epon 812 epoxy resin. After cell embedment and resin polymerization (60 ºC, 48 h), sections with a thickness of 50 nm were cut with an ultramicrotome UCT6 (Leica Microsystems, Vienna) and placed on TEM grids (Formvar carbon-coated Cu grids). Finally, the grids were further contrasted with uranyl acetate and lead citrate. All electron micrographs were obtained with a Jeol JEM 1010 MT electron microscope (Jeol, Japan) operating at 80 kV. Images were recorded with AnalySIS (SIS, Munster, Germany) on a Megaview III CCD camera.

Inhibition of p38 MAPK in mice

The p38α inhibitor PH797804 (Hope et al., 2009) was obtained from Selleckchem (#S2726) and was dissolved in 0.5% Methyl cellulose (Sigma #M7140) and 0.025% Tween 20 (Sigma #P1379) at a concentration of 1 mg/ml. From this stock solution, a dose of 10 mg/kg body weight was administered daily to mice by oral gavage for 12 consecutive days. Control mice were similarly administered with vehicle (0.5% Methyl cellulose and 0.025% Tween 20).

Bromodeoxyuridine incorporation

Bromodeoxyuridine (BrdU) is a thymidine analogue which is incorporated into newly synthesized DNA of replicating cells during S-phase of cell cycle. To detect proliferation in tissues, BrdU (Roche #10280879001) was injected intraperitoneally (1 mg /10 g body weight) 2 h before killing the mice. Incorporated BrdU was detected by immunostaining using anti-BrdU antibody, thus indicating cells that were actively replicating their DNA.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a common method to detect DNA fragmentation that results from apoptosis by labeling the terminal end of nucleic acids. To detect apoptosis in paraffin-embedded samples from DSS treated mice, the Fluorescein In situ cell death detection kit (Roche) was used according to manufacturer's instructions. Images were taken in Nikon E800 upright microscope using appropriate fluorescence filters.

Histological analysis for colon

Sample preparation

For histological analysis colon tissue was fixed in 10% formalin solution at RT overnight. The samples were then washed with PBS and kept in a Leica tissue processor for tissue dehydration. After tissue dehydration samples were embedded in paraffin blocks using the Paraffin embedding module from Leica. Colon tissues were sectioned with a microtome at 4-6 μm thickness and stained with hematoxylin and eosin (H&E) or used for immune-staining.

Hematoxylin & Eosin staining

For histological analysis, formalin-fixed and paraffin-embedded colon sections of 5 μm thickness were de-wax in xylol for 10-15 min and then rehydrated in descending series of ethanol solutions (100%, 95%, 75%, 50% and then in water) . The de-wax, rehydrated colon sections were stained with H&E using standard protocol and analyzed by pathologists in blinded fashion.

Epithelial damage and inflammation was determined using the following scoring systems. For epithelial damage: 1- intact crypts, 2- basal one-third damaged, 3- basal two-thirds damaged, 4- damaged surface epithelium. For severity of inflammation: 1- rare cells in mucosa, 2- increased cells in lamina propria, 3- confluence of cells in the submucosa, 4- transmural inflammation.

Immunohistochemistry

For immunohistochemical (IHC) staining, tissue sections on slides were de-waxed and re-hydrated as described above. After washing with tap water for 5 min, endogenous peroxidase activity was blocked for 15 min at RT in peroxidase blocking buffer. Thereafter slides were washed in tap water and antigen unmasking was performed. After antigen retrieval, slides were washed with PBS and unspecific background was blocked with blocking buffer (10% normal goat serum and 0.3% Triton x-100 in PBS). Diluted primary antibodies were added as indicated in **Table 3**. After washing the primary antibody, HRP conjugated secondary antibodies were added (**Table 4**) to the samples and then signals were visualized with DAB (3,3-diaminobenzidine), using hematoxylin as a counterstaining. To detect goblet cells, colon sections were stained with Periodic acid-Schiff (PAS) reagent. Tissue was mounted with DPX mounting medium after washing with PBS or dehydrated in descending series of ethanol solutions, cleared in xylol.

Table 3: Primary antibodies and conditions for immunostainings.

Antibody	Company	Dilution/ Incubation	Antigen retrieval
Ki67	Novocastra (NCL-Ki67-P)	1:500, 1h RT	Citrate Buffer
Cleaved casp-3	Cell signaling (9661)	1:200, 1h RT	Citrate Buffer
F4/80	eBiosciences (14-4801)	1:50, 2h RT	Proteinase-K
γ-H2AX	Millipore (05-636)	1:600, 2h RT	Citrate Buffer
Chg-A	Abcam (151601)	1:1000 o/n 4ºC	Tris-EDTA
p38α	Cell Signaling (9218)	1:50, 2h RT	Citrate Buffer
anti-BrdU	BD Biosciences (347580)	1:100 1h RT	Citrate Buffer

Table 4: HRP conjugate secondary antibodies and conditions for immunostainings.

Antibody	Company	Reference	Dilution/incubation
anti-rabbit IgG	ImmunoLogic	DPVR110HRP	45 min at RT
anti-mouse IgG	Dako	P0447	1:100, 30 min at RT
anti-rat IgG	Dako	P0450	1:75, 30 min at RT

Isolation of colon epithelial cells

Mice were sacrificed and colons were dissected, opened longitudinally and washed extensively in cold PBS. Colons were then cut into 3-4 pieces and incubated in 8 mM EDTA in PBS at 37ºC. After 15 min, EDTA solution was replaced with cold PBS and shacked vigorously for 45 sec to 1 min. This process was repeated once. Supernatants from both incubations were combined, centrifuged at 1200 rpm for 5 min at 4ºC, and pelleted cells were resuspended in 5 ml of cold PBS and centrifuged at 12000 rpm for 5 min. Pellets contain mainly IECs and were frozen or processed for protein extraction.

Isolation of peritoneal macrophages

Mice were sacrificed and a small incision was made in abdominal skin of the mouse. Carefully skin was pulled down with both hands without breaking peritoneum. Once peritoneum is visible, 5 ml cold PBS was injected into peritoneal cavity. Mouse was then swish around to wash peritoneal cavity with the injected PBS. PBS containing macrophages was then recovered from peritoneal cavity using syringe. This process was repeated once. Macrophages were pelleted at 1200 rpm for 5 min, resuspended in 3 ml DMEM containing 10% FBS and 1% penicillin-streptomycin and plated in 6 cm tissue culture plate. Next day, cells were washed 2-3 times with cold PBS to remove all dead and non-adherent cells. Adherent cells are mainly macrophages and were used for protein extraction.

Preparation of protein extracts from isolated cells and tissue

Isolated IECs or macrophages were lysed in lysis buffer for 30 min on ice. Lysed cells were centrifuged at 13000 rpm for 10 min at 4ºC and the supernatant was collected in new tube. Protein extracts from tissues were prepared in the same lysis buffer using the Precellys homogenization and lysis instrument (Bertin technologies). After homogenization, protein extracts were recovered by centrifugation at full speed for 10 min at 4ºC.

Determination of protein concentration

Total protein was quantified using the DC™ protein assay (Bio-Rad) according to manufacturer instructions. Protein concentrations were measured with a spectrophotometer at an absorbance of 750 nm. For the calculation of protein concentrations a BSA-standard curve was used.

Western blotting

After protein concentration quantification, 40 µg of total protein was boiled in 1x Laemmli sample loading buffer for 5 min and separated by electrophoresis on 10% or 12% SDS-PAGE depending on the molecular weight of the proteins of interest. After the electrophoresis, separated proteins in the gel were transferred to nitrocellulose membrane (Whatman #10401396) using a wet-blotting transfer system (Bio Rad). Ponceau red staining was used to confirm the efficiency of transfer. Ponceau red was washed with PBS and the nitrocellulose membrane was blocked, in 5% non-fat milk and 1% BSA in PBS for 1 h at RT, to reduce the unspecific binding of antibodies. After washing with PBS, membranes were incubated at 4ºC overnight with the primary antibodies, washed again with PBS and incubated with Alexa Fluro 680 or 800-conjugated secondary antibodies for 1 h at RT before visualization using the Odyssey Infrared Imaging System (Li-Cor, Biosciences). Anti-tubulin was used as a loading control. The primary and secondary antibodies and the dilutions used are listed in **Tables 5 and 6**.

Table 5: List of primary antibodies and dilutions used for western blotting.

Antibody	Company	Reference	Dilutions
p38α	Cell Signaling	9218	1:1000
p38α	Santa Cruz	sc-535	1:1000
p38γ	Homemade*	-	1:500
p38δ	Homemade*	-	1:500
phospho-p38	Cell Signaling	9211	1:600
phospho-HSP27	Cell Signaling	2401	1:600
HSP27	Santa Cruz	sc-1049	1:1000
phospho-Stat3	Cell Signaling	9145	1:600
Stat3	Cell Signaling	9132	1:1000
phospho-IKBα	Cell Signaling	9246	1:600
IKBα	Cell Signaling	4812	1:1000
Mcl-1	Cell Signaling	5453	1:1000
Bcl-2	Cell Signaling	2870	1:1000
Bak	Cell Signaling	3814	1:1000
Bax	Cell Signaling	2772	1:1000
Bcl-XL	Santa Cruz	sc-8392	1:1000
phospho-AKT	Cell Signaling	9271	1:1000
phospho-ERK1/2	Cell Signaling	9101	1:1000
phospho-JNK	BD Biosciences	612541	1:600
JNK	Santa Cruz	sc-571	1:1000
phospho-p53 Ser-15	Cell Signaling	9284	1:1000
p53	Cell Signaling	2524	1:1000
p85 PARP	Promega	G734	1:500
Caspase 3	Cell Signaling	9665	1:1000
COX-2	Santa Cruz	sc-19999	1:1000
NFκB p65	Santa Cruz	sc-372	1:1000

PCNA	Santa Cruz	sc-9857	1:1000
γ-H2AX	Millipore	05-636	1:1000
ZO-1	Invitrogen	40-2200	1:1000
Claudin-1	Invitrogen	374900	1:500
Tubulin	Sigma	T9026	1:5000

*p38γ and p38δ sheep antibodies were kindly provided by Dr. Ana Cuenda, CNB-CSIC, Madrid.

Table 6: List of secondary antibodies and dilutions used for western blotting.

Antibody	Company	Reference	Dilutions
Goat IgG (Alexa Fluor 680)	Invitrogen	A21084	1:5000
Mouse IgG (Alexa Fluor 680)	Invitrogen	A21057	1:5000
Mouse IgG (Alexa Fluor 800)	Rockland	610-731-124	1:5000
Rabbit IgG (Alexa Fluor 680)	Invitrogen	A21076	1:5000
Rabbit IgG (Alexa Fluor 800)	Rockland	611-131-122	1:5000
Sheep IgG (Alexa Fluor 680)	Invitrogen	A-21102	1:5000

IL-6 in blood and colon tissue

Blood was collected by cardiac puncture and serum samples were obtained using SST tubes (BD #365968). IL-6 concentrations in serum were determined by the CBA mouse IL-6 flex set (BD #558301) according to manufacturer's instructions.

To determine IL-6 levels in colonic tissue, 100 μg of freshly lysed colon tissue were analyzed by ELISA using anti-mouse IL-6 purified capture antibody (eBioscience #14-7060-81) and anti-mouse IL-6 biotin detection antibody (eBioscience #13-7062-81). Mouse IL-6 recombinant protein (eBioscience #14-8061-62) was used to make standard curve.

Analysis of epithelial cells and leukocytes from colon

Acute colitis was induced by treating mice with 2% DSS for 5 days. One day after termination of the DSS treatment, mice were sacrificed and colons were dissected,

opened longitudinally and washed extensively in cold PBS. Colons were then cut into 3-4 pieces and incubated in 8 mM EDTA solution in PBS at 37ºC for 15 min. After 15 min, EDTA solution was replaced with cold PBS and shacked vigorously for 45 sec. This process was repeated once. Supernatants from both incubations were combined, centrifuged at 1200 rpm for 5 min at 4ºC, and pelleted cells were digested with Dispase II (0.5 mg/ml, Roche #04942078001) at 37ºC for 25 min. After the incubation, the digested cell suspension was gently syringed with a 18G needle in order to get enriched single cell population and sequentially passed through 100, 70 and 40 μm mesh filters (BD Biosciences). Filtered cells contain epithelial cells and intra-epithelial leukocytes. To obtain lamina propria leukocytes, colon pieces were collected after EDTA incubations, cut into small pieces (2-3 mm) and digested with a mix of collagenase A (1.75 mg/ml, Roche #10103586001) and DNase I (0.05 mg/ml, Sigma #D4263) at 37ºC for 45 min. After digestion, the cell suspension was filtered as above to get a single cell suspension containing lamina propria leukocytes.

To quantify immune cell populations, single cell suspensions from both purifications were co-stained with pan-leukocyte antigen CD45 and with specific leukocyte population markers (CD11b, Gr1 and CD19) and analyzed on FACS Aria 2.0 (BD Biosciences). FITC-EpCAM (Santa Cruz #sc-53532) staining was used to discard epithelial cells by negative selection.

To get epithelial cells and total leukocytes from the colon, after induction of acute colitis whole colon tissue was digested in a mix of collagenase and DNase I, syringed and sequentially filtered as described above. Then cells were co-stained with FITC-EpCAM and APC-CD45 (BD #559864). Stained cells were analyzed and sorted for $EpCAM^+$ epithelial cells and $CD45^+$ leukocytes. RNA was isolated from sorted cells using standard TRIzol method for RT-PCR analysis.

RNA extraction from isolated cells and tissues

Total RNA was extracted from distal colon segments or isolated cells using TRIzol (Invitrogen) or Purelink RNA minikit (Ambion #12183018A) following the manufacturer's instructions. After extraction total RNA was treated with DNase I

(Roche #04716728001) to eliminate DNA contamination and RNA was repurified by the standard Phenol/Chloroform method.

RNA concentration and purity was determined by measuring the absorbance (optical density, OD) at 260 nm in relation to the absorbance at 280 nm (OD_{260}/OD_{280} ratio) using NanoDrop 2000 spectrophotometer (Thermo Scientific).

cDNA synthesis

Total RNA (1-2 μg) was reverse transcribed using a Super script II Reverse Transcriptase (Invitrogen #18064-014) and Random primers (Invitrogen #48190-011) in a final volume of 20 μl according to the manufacturer's instructions.

Quantitative real-time PCR

The cDNA was diluted 1/12 and 4 μl diluted cDNA was used in triplicate for the real-time PCR using SYBR green (Bio-Rad #1708886) in 20 μl total volume on a Bio-Rad C1000 thermal cycler machine. Each primer (**Table 7**) was used at final concentration of 0.25 μM. PCR conditions are as follows: 50ºC for 2 min, 95ºC for 10 min, 40 cycles of denaturation at 95ºC for 15 s, annealing at 56ºC for 15 s, elongation at 72ºC for 60 s, and three final steps of 95ºC for 15 s, 60ºC for 2 min and 95ºC for 15 s.

Relative quantities (Δ cycle threshold values) were obtained by normalizing against GAPDH.

Table 7: Primers used in quantitative RT-PCR

Gene (mouse)	Sequence (5′-3′)
GAPDH	FW: CTTCACCACCATGGAGGAGGC
	RV: GGCATGGACTGTGGTCATGAG
IL-6	FW: AGTTGCCTTCTTGGGACTGA
	RV: CAGAATTGCCATTGCACAAC
IL-1α	FW: GAGAGCCGGGTGACAGTATC
	RV: TGACAAACTTCTGCCTGACG

TNF-α	FW: CGTCAGCCGATTTGCTATCT
	RV: CGGACTCCGCAAAGTCTAAG
COX2	FW: AAAAGCTGGGAAGCCTTCTC
	RV: AAGTGCTGGGCAAAGAATGC
p38α (exon-2)	FW: GCATCGTGTGGCAGTTAAGA
	RV: GTCCTTTTGGCGTGAATGAT
p38α (exon-12)	FW: GCCCTCCCTCACTTCAGGAG
	RV: TGTGCTCGGCACTGGAGACC
p38α	FW: CTGACCGACGACCACGTTC
	RV: CTTCGTTCACAGCTAGGTTGC
p38β	FW: TACCATGACCCTGACGATGA
	RV: TCCTTGGCCTCAACACTTTC
p38δ	FW: GTCTGTTGGTTGCATCATGG
	RV: TCCTTGCCCTTGAAGAGTGT
p38γ	FW: TGAGTTTGTTCAGAAGCTACAGAGT
	RV: ACAGCCTGAGGGCTTGCGTT
Chg A	FW: AAGTGCGTCCTGGAAGTCATCTC
	RV: GCTTGGCTTTTCTGGCTTGC
Ki67	FW: GTGCTGACCCTGATGGGGAAGG
	RV: GCTCTTGCCCTGCCTGACACC
CyclinD1	FW: CTGCAAATGGAACTGCTTCTGGTGA
	RV: AGCAGGAGAGGAAGTTGTTGGGGCT
Muc2	FW: GCCCGTGGAGTCGTACGTGC
	RV: TTGGGGCAGAGTGAGGCGGT
Tff3	FW: TAATGCTGTTGGTGGTCCTG
	RV: CAGCCACGGTTGTTACACTG
TGFβ1	FW: GGAGGTACCGCCCGGCCCGC
	RV: GACAGCAATGGGGGTTCGGG
IL-10	FW: CCAAGCCTTATCGGAAATGA

	RV: TTTTCACAGGGGAGAAATCG
IL-12p40	FW: AGGTCACACTGGACCAAAGG
	RV: TGGTTTGATGATGTCCCTGA
ZO-1	FW: GCCGCTAAGAGCACAGCAA
	RV: GCCCTCCTTTTAACACATCAGA
Occludin	FW: TTGAAAGTCCACCTCCTTACAGA
	RV: CCGGATAAAAAGAGTACGCTGG

Analysis of apoptosis in human cancer cell lines

Human colon cancer cell lines SW-620 and Caco-2 were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% l-glutamine, and 1% penicillin-streptomycin. The p38 MAPK inhibitors SB203580 (10 μM), PH797804 (1 μM) and Birb0796 (200 nM) were added every 48 h to the cell cultures. After 96 h, all the cells (including floating cells in the medium) were collected and lysed. Cell lysates were analyzed by western blotting using p85 PARP antibody (Promega) as an apoptosis marker.

Statistical methods

Data are presented as mean ± SEM. Statistical significance was determined by Student's *t* test using GraphPad Prism 4 software. *P* values less than 0.05 were considered statistically significant.

Results

Downregulation of p38α in IECs

In order to study the role of p38α in colitis and CAC, we generated mice lacking p38α in IECs by crossing p38α (lox/lox) mice (Heinrichsdorff et al., 2008) with Villin-Cre mice (el Marjou et al., 2004), which express Cre recombinase under the control of the promoter for the IEC-specific gene villin. We confirmed the efficiency of p38α deletion in the whole colon lysates and in isolated colon epithelial cells by western blotting. The remaining p38α expression in whole colon lysates may be due to the presence of non-epithelial cells in colon. The downregulation of p38α was more evident in isolated colon epithelial cells **(Figure 7A)**. p38α downregulation in IECs did not affect the expression of other p38 MAPKs as determined by western blotting and by quantitative real-time PCR **(Figure 7B and 7C)**. Mice deficient of p38α in IEC (p38α-Δ^{IEC}) appeared healthy and showed no obvious phenotype.

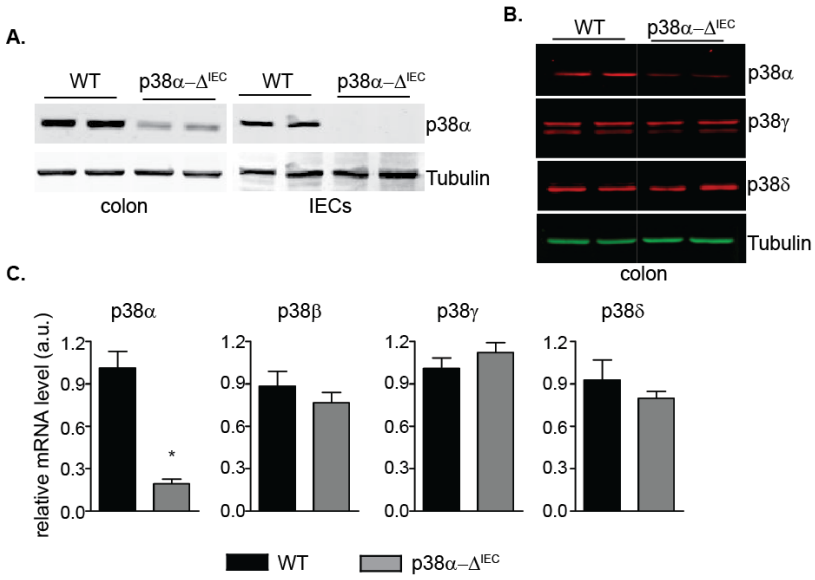


Figure 7. Analysis of p38α downregulation in IECs.

(A) Western blotting of p38α in whole colon lysates and in isolated IECs (colon crypts) from non-treated WT and p38α-Δ^{IEC} mice.

(B) Western blotting of p38 MAPKs in the whole colon lysates from WT and p38α-Δ^{IEC} mice. Tubulin was used as a loading control.

(C) Relative mRNA expression levels of p38 MAPKs in the distal colon of non-treated WT and p38α-Δ^{IEC} mice were determined by qRT-PCR and normalized to GAPDH mRNA. Data are means ± SEM (n = 4) ∗, $p<0.05$.

Downregulation of p38α in IECs increases CAC

To investigate the role of p38α in CAC, we used a protocol that combines the carcinogen AOM with DSS-induced colitis. When we applied this protocol to p38α-Δ^{IEC} mice or to their wild-type (WT) littermates **(Figure 8A)**, all mice developed colon tumors 100 days after the AOM injection. We confirmed that p38α expression was significantly downregulated in the colon lysates from AOM/DSS-treated p38α-Δ^{IEC} mice **(Figure 8B)**. The tumors were mainly distributed in the distal to middle colon **(Figure 8C)**, which is the predominant localization of colorectal human tumors (Neufert et al., 2007). No tumors were found in the proximal part of the colon in either group of animals. We noticed that p38α-Δ^{IEC} mice had more macroscopic tumors than WT mice, but the average tumor size was not significantly changed **(Figure 8D)**. On the other hand, the average tumor load, calculated by adding up the diameters of all the tumors in a given mouse, was also significantly higher in p38α-Δ^{IEC} mice **(Figure 8D)**.

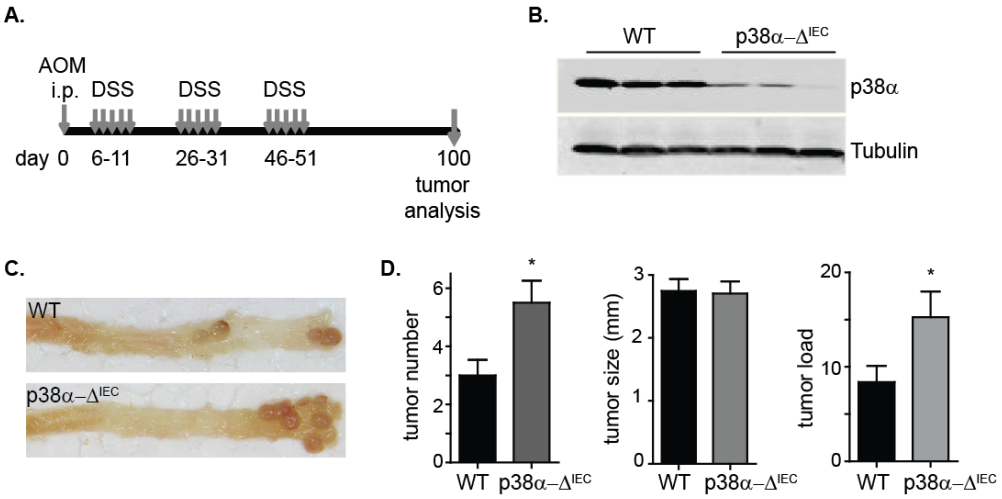


Figure 8. Downregulation of p38α in IECs increases susceptibility to AOM/DSS-induced colon tumorigenesis.

(A) Schematic representation of the AOM/DSS protocol for colorectal tumorigenesis.

(B) Western blotting of p38α in colon lysates from AOM/DSS-treated WT and p38α-Δ^{IEC} mice. Tubulin was used as a loading control.

(C) Representative images of colons from WT and p38α-Δ^{IEC} mice showing tumors in the distal/middle region.

(D) Average number and size of tumors formed in WT and p38α-Δ^{IEC} mice at the end of the AOM/DSS protocol. Average tumor load was determined by adding all tumor diameters for a given animal. Data represent means ± SEM (n ≥ 7). *, p<0.05.

Next we histologically analyzed the tumors developed in WT and p38α-Δ^{IEC} mice upon AOM/DSS treatment. Colon sections were H&E stained and microscopic tumors were counted and graded into low- and high- grade tumors. We found more tumors of both low-grade (13 vs. 7 in WT, n=8) and high-grade (29 vs. 18 in WT, n=8) in p38α Δ^{IEC} mice **(Figure 9A)**, but the relative proportion of low and high-grade tumors detected by microscopy in p38α-Δ^{IEC} versus WT mice was similar as in the macroscopic analysis. These observations indicate that p38α may play a role in colon tumor initiation.

We also analyzed the levels of cell proliferation and apoptosis in the colon tumors from WT and p38α-Δ^{IEC} mice at day 100 after AOM injection. Colonic sections were stained with antibodies against BrdU or cleaved Caspase 3. Quantification of BrdU positive proliferative cells and Cleaved caspase 3 positive apoptotic cells revealed no differences in the tumors from WT and p38α Δ^{IEC} mice **(Figure 9B)**.

Chronic inflammation induced by repeated cycles of DSS is a main contributor of colon tumorigenesis induced by AOM/DSS, and inflammatory mediators such as COX-2, IL-6, IL-1α and TNF-α are upregulated in these tumors. We found no significant differences in the basal mRNA expression levels of inflammatory mediators between WT and p38α-Δ^{IEC} mice before treatment (see below **Figure 14A**). However, the colon from p38α-Δ^{IEC} mice treated with AOM/DSS showed higher COX-2 and IL-6 mRNA levels than the colon from treated WT mice **(Figure 10A)**. Interestingly, circulating IL-6 levels were also higher in serum from p38α-Δ^{IEC} mice than in WT mice at the end of the AOM/DSS protocol **(Figure 10B)**. This is consistent with the known importance of IL-6 for colon tumor development.

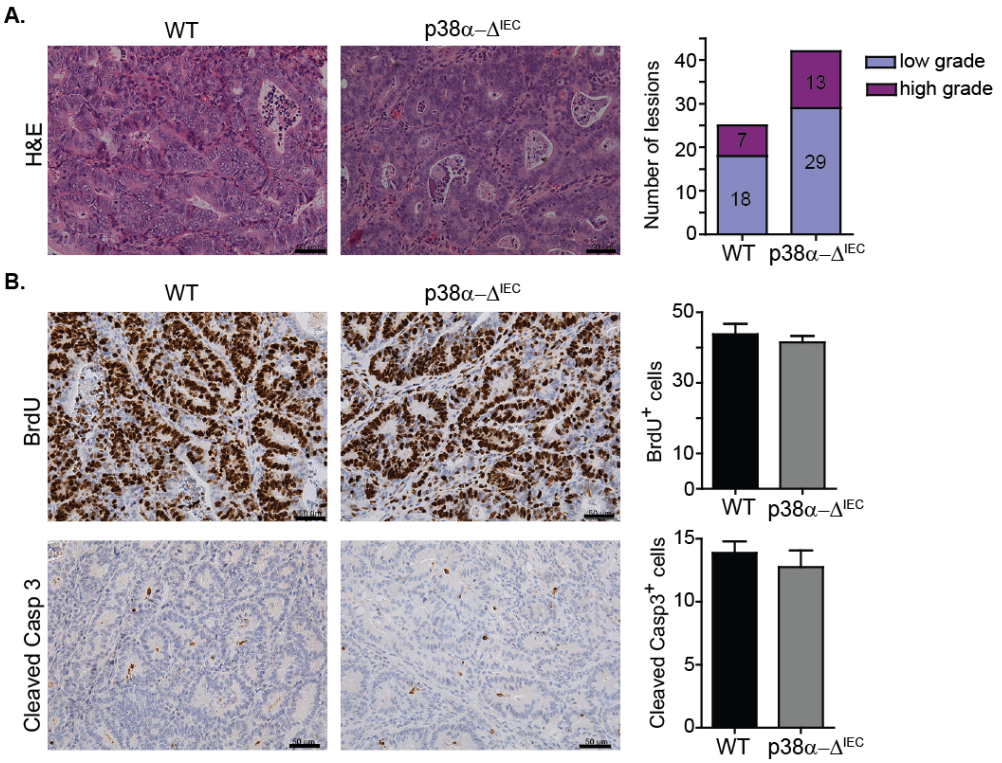


Figure 9. Downregulation of p38α in IECs does not affect proliferation and apoptosis within tumors induced by AOM/DSS.

(A) Representative images of H&E stained colon tumors at the end of the AOM/DSS protocol. Tumors were microscopically analyzed and classified into low or high grade (n=8).

(B) Colon sections from WT and p38α-Δ^{IEC} mice were stained with BrdU or Cleaved Caspase 3 to determine cell proliferation and apoptosis, respectively. Histograms show average numbers of $BrdU^+$ cells or Cleaved caspase 3^+ cells per field. Data represent means ± SEM (n = 4). Bars = 50 μm

Taking together, this data indicate that p38α in IECs suppresses CAC. Since tumor growth was not affected, we hypothesized that p38α signaling in IECs regulates tumor initiation.

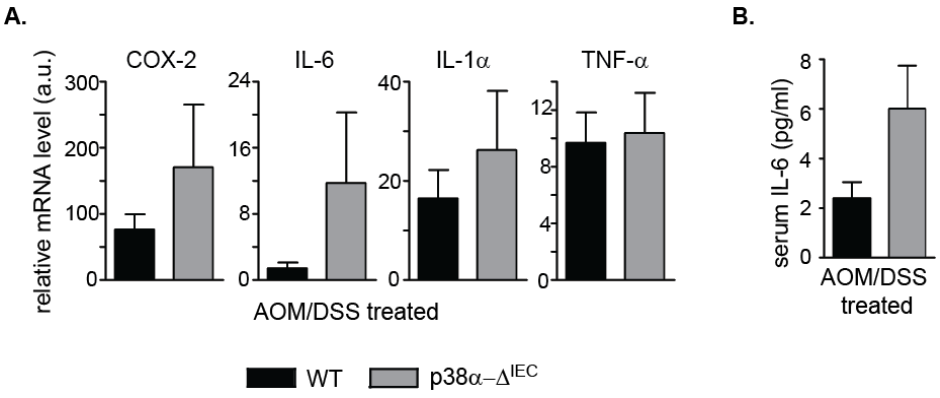


Figure 10: Expression of inflammatory mediators in AOM/DSS-treated mice.
(A) Analysis by real-time qPCR of mRNA expression levels for the indicated pro-inflammatory mediators in colon from WT and p38α-Δ^{IEC} mice at the end of the AOM/DSS protocol. Data are means ± SEM (n = 4).
(B) IL-6 protein levels in blood serum from mice after the AOM/DSS protocol. Data are means ± SEM (n = 4).

Early AOM response is not affected in p38α-Δ^{IEC} mice

We investigated if the differences in tumorigenesis between WT and p38α-Δ^{IEC} mice were due to differences in the initial response to AOM. However, AOM-induced DNA damage, as measured by γ-H2AX levels, was very similar in colon sections and colon lysates from WT and p38α-Δ^{IEC} mice **(Figure 11A and 11B).**

Alkylating agents such as AOM induce rapid p53-dependent apoptosis of IECs at the crypt base (Toft et al., 1999), but we observed no differences in apoptosis between colons from WT and p38α-Δ^{IEC} mice after AOM injection **(Figure 11C)**. Consistent with this, we observed no differences in the phosphorylation of p53 at Ser-15 **(Figure 11B).** This phosphorylation is induced in response to DNA damage and leads to cell cycle arrest or apoptosis (Al Rashid et al., 2005; Long et al., 2007; Takeba et al., 2007). Altogether, these results indicate that p38α downregulation in IECs does not affect the initial response to AOM.

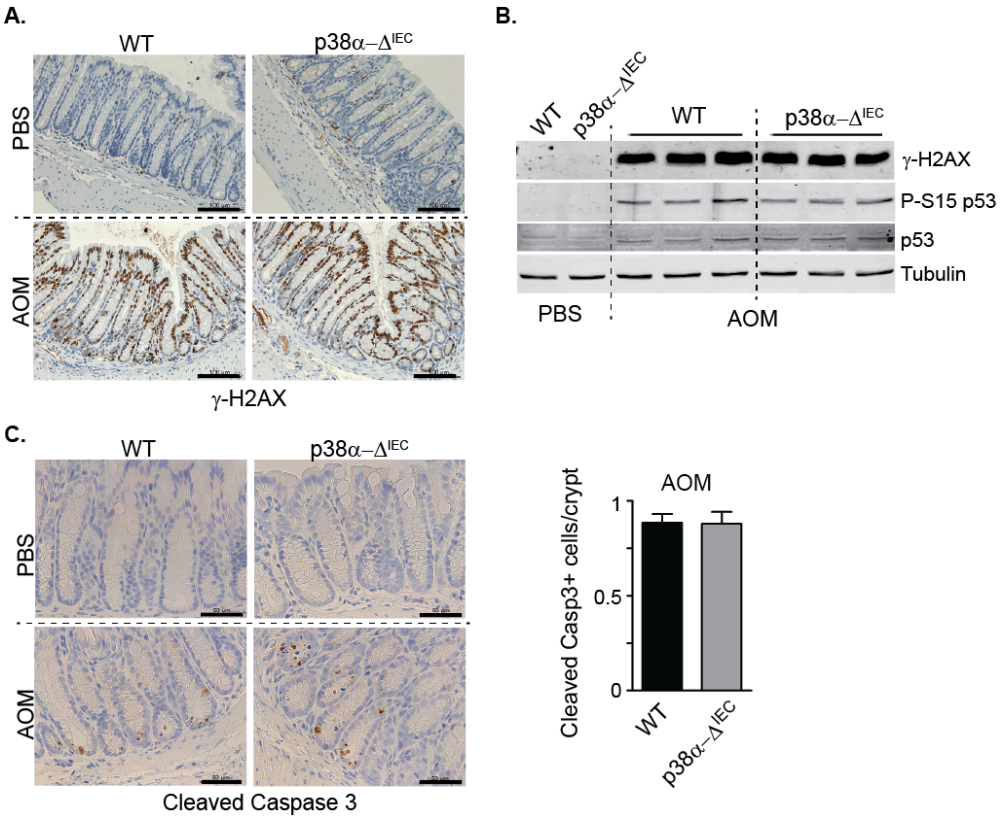


Figure 11. Downregulation of p38α in IECs does not affect AOM-induced DNA damage.
WT and p38α-Δ^{IEC} mice were intraperitoneally injected with AOM or PBS and killed 7 h later.
(A). Colon sections from WT and p38α-Δ^{IEC} mice were stained with γ-H2AX antibody to detect AOM-induced DNA damage. Bars = 100 μm.
(B) Colon lysates from AOM-treated WT and p38α-Δ^{IEC} mice were analyzed by western blotting using the indicated antibodies.
(C). Colon sections from WT and p38α-Δ^{IEC} mice were stained with cleaved caspase 3 antibody to detect apoptosis. Bars = 100 μm. The average number of apoptotic cells (Cleaved caspase 3^{+}) at the base of crypts in AOM-treated mice was quantified. Data represent means ± SEM (n =3).

p38α-Δ^{IEC} mice are more susceptible to DSS-induced colitis

The AOM/DSS tumorigenesis protocol is strongly dependent on the inflammation caused by repeated cycles of DSS (Neufert et al., 2007). Moreover, it is well established that inflammation is a critical contributor to tumorigenesis (Hussain

and Harris, 2007). Since we did not detect differences in the initial response to AOM, we investigated if the enhanced tumorigenesis in p38α-Δ^{IEC} mice was due to differences in inflammation and epithelial damage caused by DSS. To test this, mice were administered 2% DSS for 5 days to induces acute colitis and body weight changes were recorded. Loss of body weight can be used as an indicator for the severity of DSS-induced colitis.

We found that p38α-Δ^{IEC} mice lost significantly more body weight than WT mice **(Figure 12A)**. This suggested that p38α-Δ^{IEC} mice probably had enhanced inflammation and intestinal damage. To confirm this possibility, mice were given DSS for 5 days and then were sacrificed at days 6 and 9 (one and four days after termination of the DSS treatment) to histologically analyze colon sections **(Figure 12B)**. Histological analysis revealed that DSS-treated p38α-Δ^{IEC} mice had significantly more epithelial damage in the distal-middle part of the colon at both day 6 and day 9 than DSS-treated WT mice **(Figure 12C)**.

At day 6, WT colon tissues showed minimal to mild inflammation while colon tissues from p38α-Δ^{IEC} mice showed moderate to severe inflammation with many areas of complete crypt loss and erosions **(Figure 12B)**. At day 9, WT mice had moderate inflammation with some regenerative crypts, whereas p38α-Δ^{IEC} mice showed moderate to severe inflammation **(Figure 12B)**.

These results indicate more epithelial damage and inflammation in p38α-Δ^{IEC} mice upon acute DSS- administration.

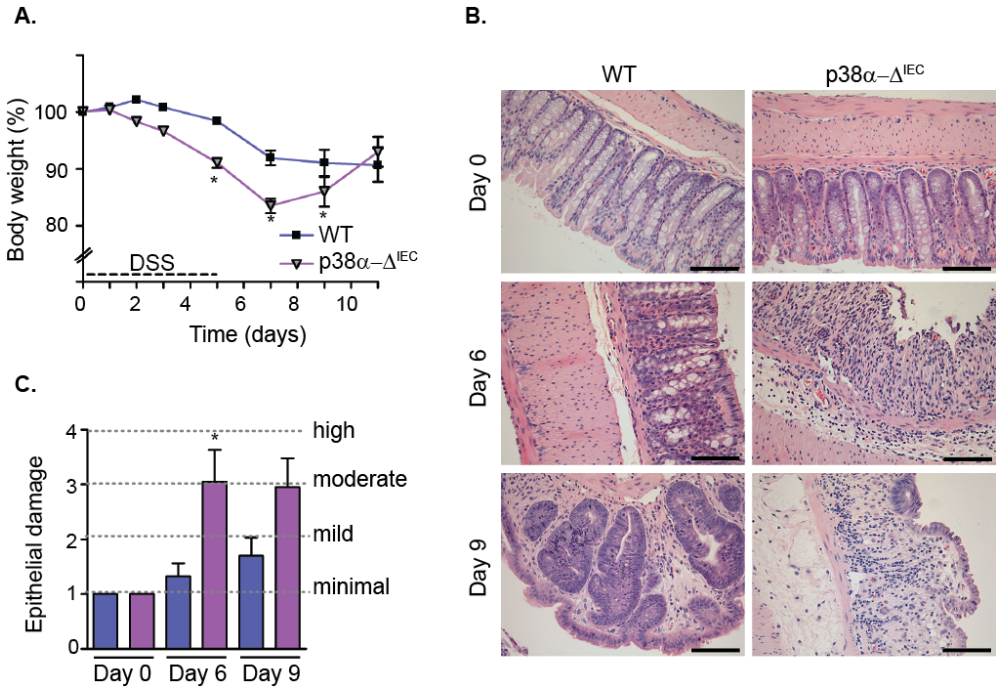


Figure 12. Downregulation of p38α in IECs increases susceptibility to DSS-induced colitis and epithelial damage.

(A) Body weight loss induced by 2% DSS in WT and p38α-Δ^{IEC} mice was recorded every other day after DSS administration. Data are means ± SEM (n ≥ 8) *, $p<0.05$.

(B) Representative H&E-stained colon sections from WT and p38α-Δ^{IEC} mice that were either untreated or treated with 2% DSS for 5 days and analyzed at days 6 and 9. Bars = 100 µm.

(C) Quantification of epithelial damage induced by DSS treatment. Data represent means ± SEM (n =4) *, $p<0.05$

Increased immune cell infiltration in p38α-Δ^{IEC} mice treated with DSS

Since p38α-Δ^{IEC} mice showed higher epithelial damage and inflammation, we characterized the immune cell infiltration in the colon of DSS-treated WT and p38α-Δ^{IEC} mice. Quantification of infiltrating cells in H&E stained tissues at day 6 (one day after termination of the DSS treatment) revealed higher infiltration in the colon of p38α-Δ^{IEC} mice **(Figure 13A).**

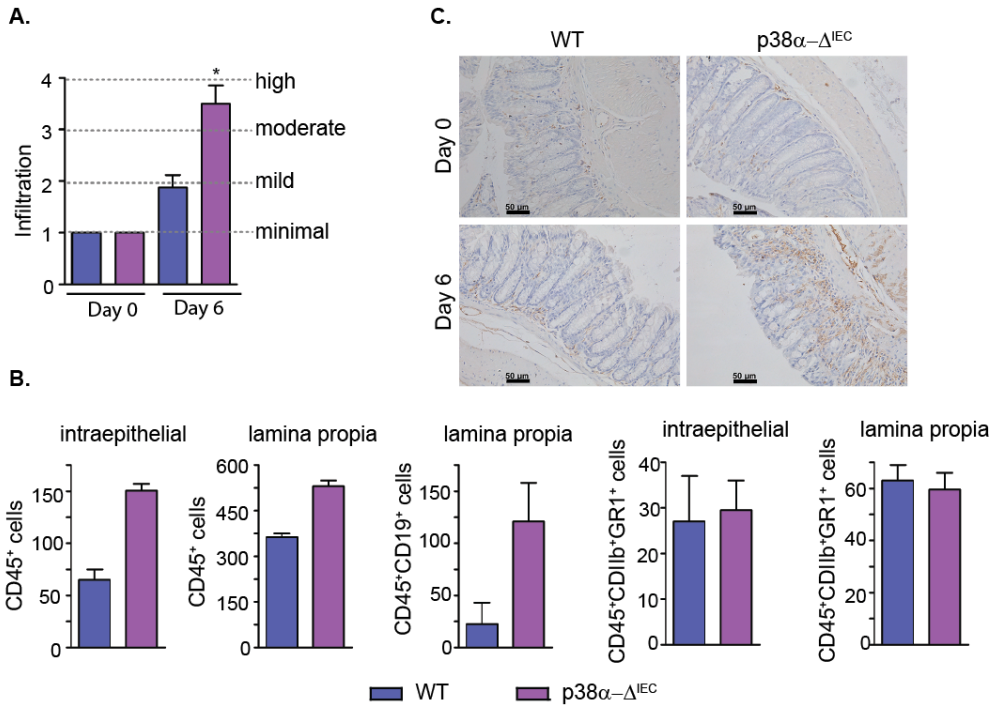


Figure 13. Increased immune cell infiltration in p38α-Δ^{IEC} mice treated with DSS.
(A) Quantification of infiltrating immune cells in H&E-stained colon sections of mice that were either untreated or treated with 2% DSS for 5 days and analyzed at day 6. Data represent means ± SEM (n =4) *, $p<0.05$.
(B) CD45$^+$ leukocytes, CD19$^+$ B-cells and Gr1$^+$ neutrophils in colon lamina propria and intraepithelial compartments of mice treated with 2% DSS for 5 days and analyzed at day 6. Histograms show numbers of the indicated cells per 10^3 total cells. Data represent means ± SEM.
(C) Colon sections from WT and p38α-Δ^{IEC} mice treated with DSS as in (A) were stained with F4/80 antibody to identify macrophages.

To characterize infiltrating immune cell populations, mice were treated with DSS for 5 days and one day later the epithelial/intraepithelial cells and lamina propria cells of the colon were separated by enzymatic digestion. Isolated cells were stained with the common leukocyte marker CD45, neutrophil-specific marker Gr1 or B cell-specific marker CD19 and quantified by flow cytometry analysis. We found significantly more CD45$^+$ cells in both epithelial/intraepithelial and lamina propria fractions from p38α-Δ^{IEC} mice compared to WT mice, and B cells (CD45$^+$CD19$^+$) also

accumulated more in the lamina propria of p38α-Δ^{IEC} mice subjected to DSS-induced colitis **(Figure 13B)**. However, there were no differences in neutrophil infiltration ($CD45^+CD11b^+Gr1^+$) between the colons from WT and p38α-Δ^{IEC} mice **(Figure 13B)**. Macrophages are important components of the immune infiltrates in DSS-treated mice and we found more macrophages in the colon of p38α-Δ^{IEC} mice compared to WT mice at day 6 **(Figure 13C)**. No differences in macrophage infiltration were observed between non DSS-treated WT and p38α-Δ^{IEC} mice.

Increased inflammatory mediators in p38α-Δ^{IEC} mice treated with DSS

Infiltrating immune cells produce cytokines and chemokines that modulate the inflammation process. We analyzed the expression levels of inflammatory mediators and found that IL-6 and COX-2 mRNAs were both significantly upregulated in the colon of DSS-treated p38α-Δ^{IEC} mice compared to WT animals, whereas IL-1α and TNFα mRNAs were comparable in WT and p38α-Δ^{IEC} mice **(Figure 14A)**.

Increased IL-6 mRNA levels in DSS-treated p38α-Δ^{IEC} mice prompted us to check IL-6 protein levels in blood serum and colon lysates from these mice. We found that the IL-6 protein concentration was significantly higher in colon from p38α-Δ^{IEC} mice compared to WT animals after DSS treatment **(Figure 14B)**. IL-6 levels were also reproducibly higher in blood from p38α-Δ^{IEC} mice **(Figure 14B)**. The increased IL-6 levels found in p38α-Δ^{IEC} mice were unexpected, since the p38α pathway is known to positively regulate IL-6 expression in several cell types (Cuenda and Rousseau, 2007). This could be due to the presence in the p38α-deficient colon of more infiltrating immune cells, which are an important source of IL-6. Alternatively, p38α could negatively regulate IL-6 production in the IECs. To test this possibility, IECs and total leukocyte populations were isolated from DSS-treated animals by FACS sorting and the expression levels for inflammatory mediators were analyzed. We found that IL-6, IL-1α and COX-2 mRNAs were all upregulated in the IECs from

DSS-treated p38α-Δ^{IEC} mice compared to WT animals **(Figure 14C)**, whereas TNFα mRNA levels did not change. In contrast, the mRNA levels for IL-6, IL-1α, COX-2 and TNFα were similar in the leukocytes from colon of WT and p38α-Δ^{IEC} mice **(Figure 14C)**, which is consistent with the leukocytes being WT in both cases. Thus, the upregulation of IL-6 and COX-2 can be probably accounted for by both the presence of more infiltrating cells in the colon of p38α-Δ^{IEC} mice and the enhanced expression of these genes in p38α-deficient IECs.

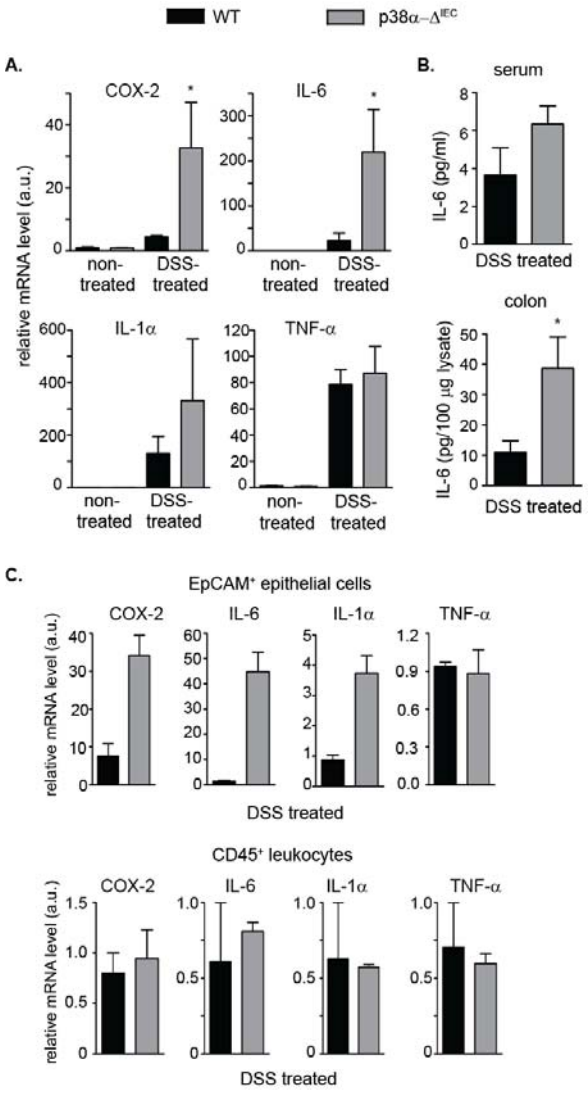


Figure 14. Elevated inflammatory mediators in p38α-Δ^{IEC} mice after DSS-induced colitis.

(A) Expression of inflammatory mediators in the distal colon of non-treated and DSS-treated WT and p38α-Δ^{IEC} mice. Relative mRNA expression levels for the indicated genes were determined by real-time qPCR and were normalized to GAPDH mRNA. Expression levels in non-treated WT and p38α-Δ^{IEC} mice were the same and were given the value of 1. Data are means ± SEM (n = 4) *, $p<0.05$.

(B) IL-6 protein levels in blood serum and whole colon lysates of DSS-treated WT and p38α-Δ^{IEC} mice were determined. Data are means ± SEM (n = 5).

(C) Expression of inflammatory mediators in Epcam⁺ epithelial cells and CD45⁺ leukocyte populations from DSS-treated WT and p38α-Δ^{IEC} mice. Relative mRNA expression for the indicated genes was determined by real-time qPCR and normalized to GAPDH mRNA.

Consistent with the increased IL-6 expression levels observed in the colon of p38α-Δ^{IEC} mice after 5 days of DSS treatment, we also detected phosphorylation and degradation of IkBα, suggesting activation of the NFkB pathway, as well as enhanced phosphorylation of STAT3, a downstream target of IL-6 family receptors. Inflammatory mediator COX-2 protein level was also elevated p38α-Δ^{IEC} mice after 5 days of DSS treatment **(Figure 15)**.

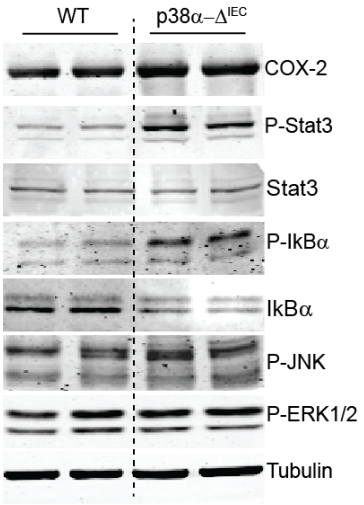


Figure 15. Elevated inflammatory mediators in p38α-Δ^{IEC} mice after DSS-treatment. Western blotting with the indicated antibodies and whole colon lysates prepared from DSS-treated WT and p38α-Δ^{IEC} mice. Colon lysates from 2 different mice were individually analyzed in each case (one per lane).

Increased apoptosis in p38α-Δ^{IEC} mice treated with DSS

DSS is believed to induce colonic inflammation through physical disruption of the mucosal barrier, inducing epithelial cell apoptosis and thus exposing inflammatory cells of the lamina propria to lumen bacteria and bacterial products (Okayasu et al., 1990). Epithelial apoptosis was also reported in UC patients, which leads to colonic inflammation (Iwamoto et al., 1996).

As we detected more inflammatory cells in the colon of DSS-treated p38α-Δ^{IEC} mice, we hypothesized that the increased inflammation in these mice could be due to enhanced epithelial cell apoptosis. To test this, we treated mice with 3% DSS for 3 days and then apoptosis was detected using TUNEL staining. Apoptosis was

detected in both WT and p38α-Δ^{IEC} mice after DSS treatment, but p38α-Δ^{IEC} mice showed more apoptosis compared to WT mice **(Figure 16 A & B)**.

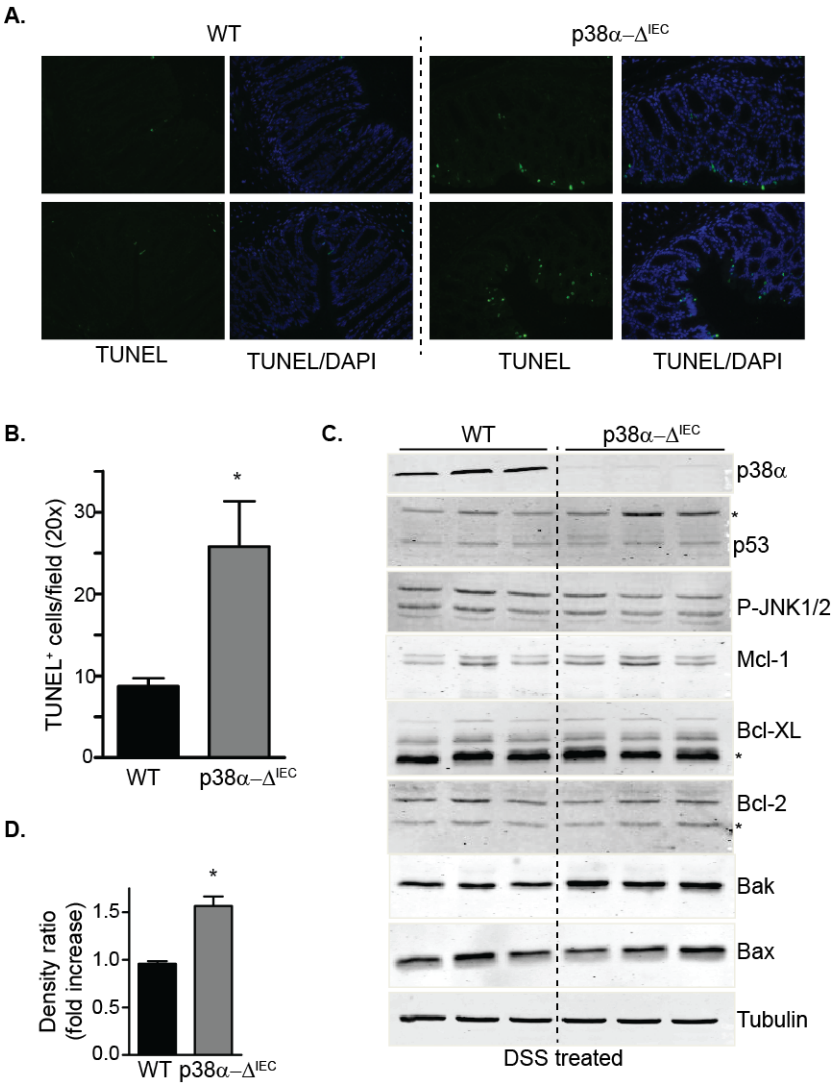


Figure 16. Downregulation of p38α in IECs results in enhanced apoptosis after DSS-induced colitis.

(A) Representative TUNEL staining of colon sections from WT and p38α-Δ^{IEC} mice 3 days after DSS treatment.

(B) Quantification of TUNEL+ apoptotic cells. Data represent means ± SEM (n =3) *, $p<0.05$.

(C) Western blotting with the indicated antibodies and colon epithelial cells obtained from DSS-treated WT and p38α-Δ^{IEC} mice. Colon lysates from 3 different mice were individually analyzed in each case (one per lane). Asterisk shows non-specific bands.

(D) Densitometric quantification of Bak normalized to tubulin in western blot. Data represent means ± SEM (n =3) *, $p<0.05$.

Biochemical analysis of colon epithelial cells isolated three days after DSS treatment confirmed that p38α was efficiently downregulated in p38α-Δ^{IEC} mice. Western blot analysis of Bcl-2 family proteins showed 1.5 fold induction of the pro-apoptotic protein Bak in p38α-Δ^{IEC} mice, whereas expression of Bax and the anti-apoptotic proteins Mcl-1, Bcl-2 and Bcl-XL were similar in WT and p38α-Δ^{IEC} mice **(Figure 16C)**. The increased expression of the pro-apoptotic protein Bak could account for the increased apoptosis observed in the p38α-Δ^{IEC} mice upon DSS treatment. Neither upregulation of p53 nor change in JNK phosphorylation was detected in p38α-Δ^{IEC} mice. These results suggest that the p53 and JNK pathways do not contribute to the increased apoptosis induced by DSS in p38α-Δ^{IEC} mice.

p38α downregulation induces hyperproliferation of IECs treated with DSS

IL-6 is an important regulator of the proliferation and survival of IECs (Bollrath et al., 2009; Grivennikov et al., 2009). Since we detected more IL-6 both in the whole colon and in IECs isolated from p38α-Δ^{IEC} mice, we analyzed cell proliferation by Ki67 immunostaining in the colon from DSS-treated WT and p38α-Δ^{IEC} mice. We treated mice for 5 days with 2% DSS and collected samples at day 6, day 9 and day 13. At day 6 (one day after DSS termination), we observed no differences in proliferation between the areas of intact crypts in WT and p38α-Δ^{IEC} mice **(Figure 17)**. In fact, proliferation was significantly decreased in both cases when compared to untreated animals, in agreement with previous reports (Boismenu et al., 2002). Interestingly, we found more proliferative cells in p38α-Δ^{IEC} than in WT mice in the areas of complete crypt loss at day 6, which probably represents a mixture of IECs and inflammatory cells **(Figure 17)**.

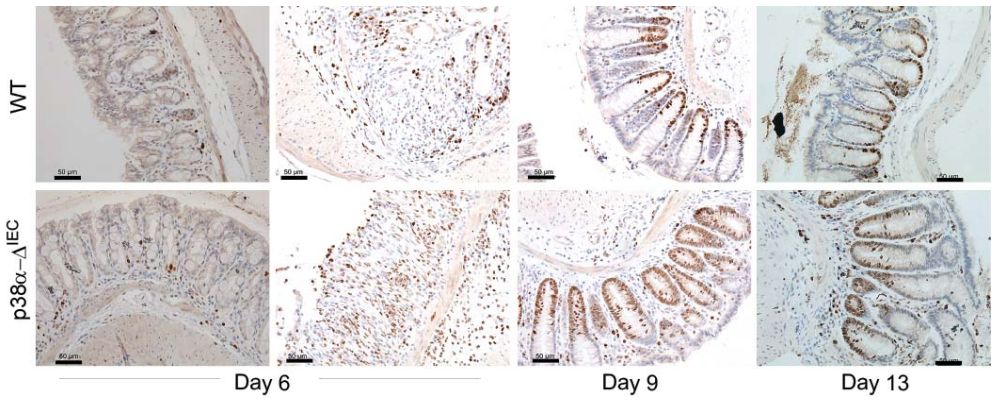


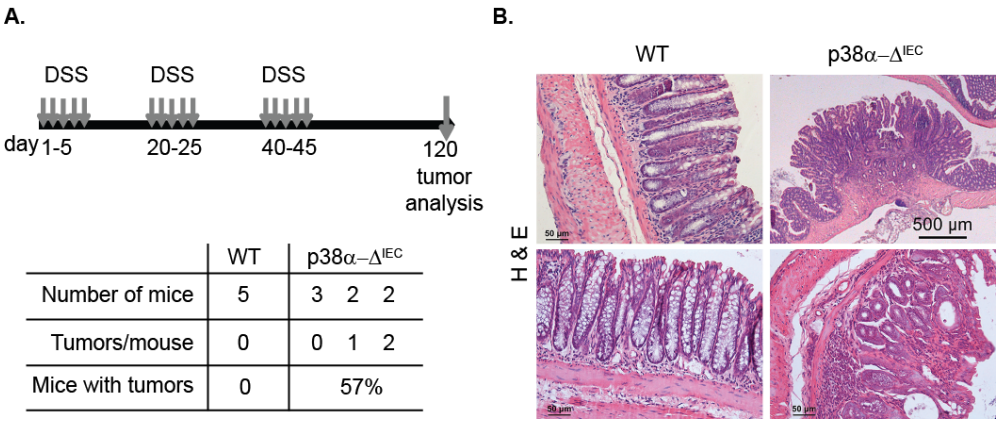
Figure 17. Loss of p38α induces hyperproliferation of IECs upon DSS treatment.
Sections from the distal colon of WT and p38α-Δ^{IEC} mice treated with 2% DSS for 5 days were stained with Ki67 antibody to detect proliferation at days 6, 9 and 13. Day 6: left panel shows an area with intact crypt architecture; right panel is an area with damage and crypt loss. Day 9: shows regenerating epithelia.

DSS is toxic to intestinal epithelial cells causing epithelial damage and inflammation. After DSS removal, damaged IECs start the repair and regeneration process. Consistent with this, we observed a higher proliferation rate in IECs at days 9 and 13 (4 and 8 days after termination of the DSS treatment) in WT and p38α-Δ^{IEC} mice compared to non-treated animals. Surprisingly, proliferation was much higher in p38α-Δ^{IEC} mice than in WT mice during the regeneration of the intestinal epithelia **(Figure 17)**. These data suggest that p38α downregulation results in the hyperproliferation of IECs after DSS-induced injury and colitis.

DSS-treatment suffices to induce colon tumors in p38α-Δ^{IEC} mice

Inflammatory cytokines are important for tissue repair, but when tissue injury and inflammation cannot be resolved due to repetitive damage, the resulting process of unchecked compensatory proliferation and regeneration can promote tumorigenesis (Kuraishy et al., 2011). To test this possibility, we treated mice with three cycles of DSS, to induce repeated injury/inflammation, but in the absence of

the AOM carcinogen **(Figure 18A)**. Macroscopic examination revealed that WT mice did not develop any tumors in the absence of AOM treatment as expected. However, about 60% of the p38α-Δ^{IEC} mice developed at least 1 tumor **(Figure 18A)**. Microscopic examination of H&E stained colon sections confirmed the presence of tumors in p38α-Δ^{IEC} mice, which also contained more aberrant crypt foci with hyperchromatism of nuclei as well as dysplastic and hyperplastic crypts compared to WT mice **(Figure 18)**.



	WT	p38α–Δ^{IEC}
Number of mice	5	3 2 2
Tumors/mouse	0	0 1 2
Mice with tumors	0	57%

Figure 18. DSS alone suffices to induce colon tumors in p38α-Δ^{IEC} **mice.**
(A) Schematic representation of the DSS protocol used to induce chronic colitis. Mice were killed at day 120 for analysis. Table shows the incidence of tumors per mouse.
(B) Representative images of H&E stained colon tumors at the end of the DSS treatment.

Next, we analyzed macrophage infiltration in the colon sections and found that WT mice had low to moderate infiltration whereas in p38α-Δ^{IEC} mice macrophage infiltration was medium to high **(Figure 19A)**. Expression levels of the inflammatory mediators COX-2, IL-6, TNF-α, and IL-12p40 were also higher in the colon of DSS-treated p38α-Δ^{IEC} mice compared to WT mice **(Figure 19B)**.

Taking together, these data suggest that repeated epithelial damage and inflammation induced by DSS in the absence of p38α, results in uncontrolled

hyperproliferation of IECs and a pro-tumorigenic environment that ultimately induces colon hyperplasia and tumor formation.

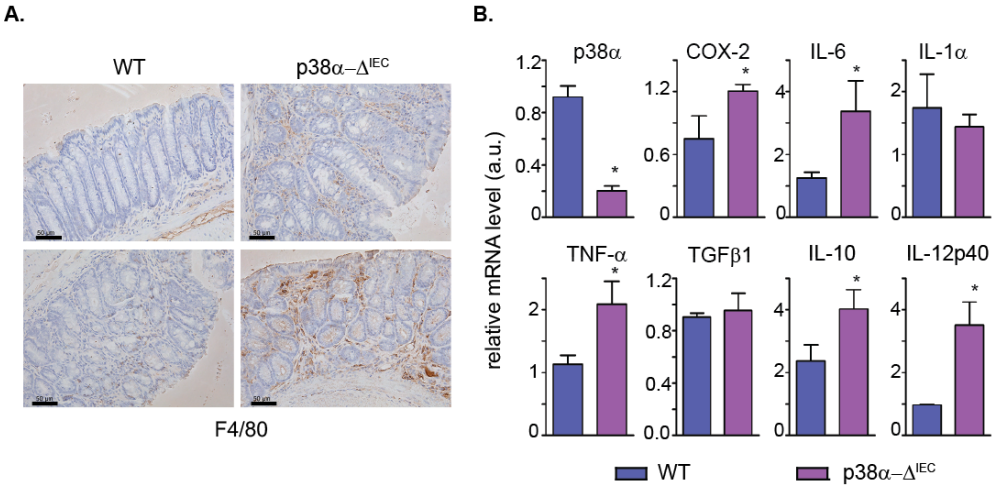


Figure 19. Increased inflammatory mediators in p38α-Δ^{IEC} mice after chronic colitis induced by DSS.

(A) Colon sections from WT and p38α-Δ^{IEC} mice treated with 3 cycles of DSS were stained with F4/80 antibody to detect macrophages.

(B) Relative mRNA expression levels of the indicated genes in the colon of WT and p38α-Δ^{IEC} mice were determined by qRT-PCR and normalized to GAPDH mRNA. Data are means ± SEM (n = 4) *, $p<0.05$.

AOM and one cycle of DSS induce aberrant crypt foci and hyperplasia in p38α-Δ^{IEC} mice

Early response to AOM alone was not very different in WT and p38α-Δ^{IEC} mice **(Figure 11)**, whereas treatment with DSS alone induced hyperproliferation of IECs in the p38α-Δ^{IEC} mice **(Figure 17)**. Moreover, we detected colon tumors in p38α-Δ^{IEC} mice after repetitive DSS-induced injury **(Figure 18)**. Therefore, we investigated if the combined treatment of AOM withy one cycle of DSS would result in more aberrant crypt foci (ACF) formation in p38α-Δ^{IEC} mice due to increased proliferation and inflammation caused by DSS. ACF are believed to be the earliest preneoplastic change that can be seen in the colonic mucosa (Pretlow et al., 1991; Pretlow et al., 1992; Takayama et al., 1998). Mice were treated with AOM and one cycle of DSS

and were killed 2 weeks after the removal of DSS, the time when the second cycle of DSS usually starts in the full AOM/DSS protocol. Analysis of H&E stained colonic sections on revealed more hyperplastic and dysplastic ACFs in p38α-Δ^{IEC} mice **(Figure 20)**. These results are in agreement with the higher number of colon tumors developed in p38α-Δ^{IEC} mice after the full AOM/DSS protocol.

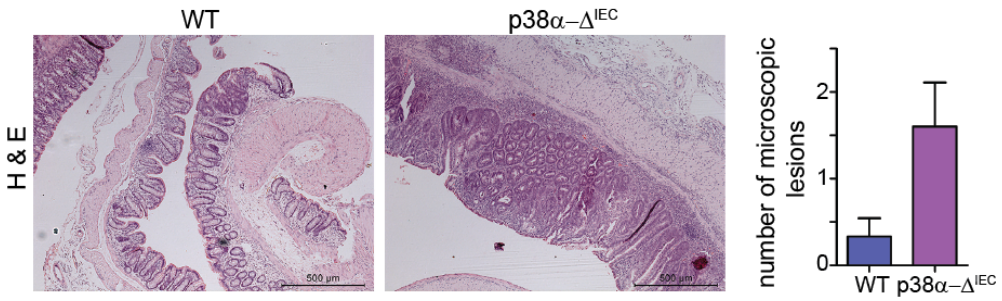


Figure 20. p38α deficiency in IEC increases susceptibility to AOM and one cycle of DSS. Representative H&E-stained colon sections from WT and p38α-Δ^{IEC} mice that were treated with AOM and 2% DSS for 5 days and analyzed after 2 weeks of DSS termination. Numbers of microscopic lesions were quantified. Data represent means ± SEM (n ≥4).

p38α regulates intestinal homeostasis

Altered epithelial barrier function has been associated with IBD and intestinal inflammation in humans (Westbrook et al., 2010). Since p38α-Δ^{IEC} mice show more body weight loss, epithelial damage, inflammation and infiltration after DSS induced acute colitis in comparison to their WT littermates, we hypothesized that p38α-Δ^{IEC} mice could have altered intestinal homeostasis.

As in other epithelial tissues, intestinal homeostasis is the result of a fine balance between cell proliferation and differentiation. We analyzed the effect of p38α downregulation in IEC proliferation and differentiation. In agreement with a recent report (Otsuka et al., 2010), we found that p38α downregulation in IECs had no effect on gross morphology of the H&E stained colon tissue. However, p38α-Δ^{IEC} mice had more Ki67^{+} proliferative cells in the distal colon than the WT littermates **(Figure 21A)**. We analyzed the two main types of differentiated cells in the colon by

IHC and found significantly less chromogranin A$^+$ (ChgA$^+$) enteroendocrine cells in p38α-Δ^{IEC} mice compared to WT littermates, while the number of periodic acid schiff$^+$ (PAS$^+$) goblet cells was also reduced in p38α-Δ^{IEC} mice but did not reach a statistically significant value **(Figure 21A)**. Mucins produced by goblet cells, form a protective mucous layer, which serves as a first barrier to pathogens and chemically induced injury.

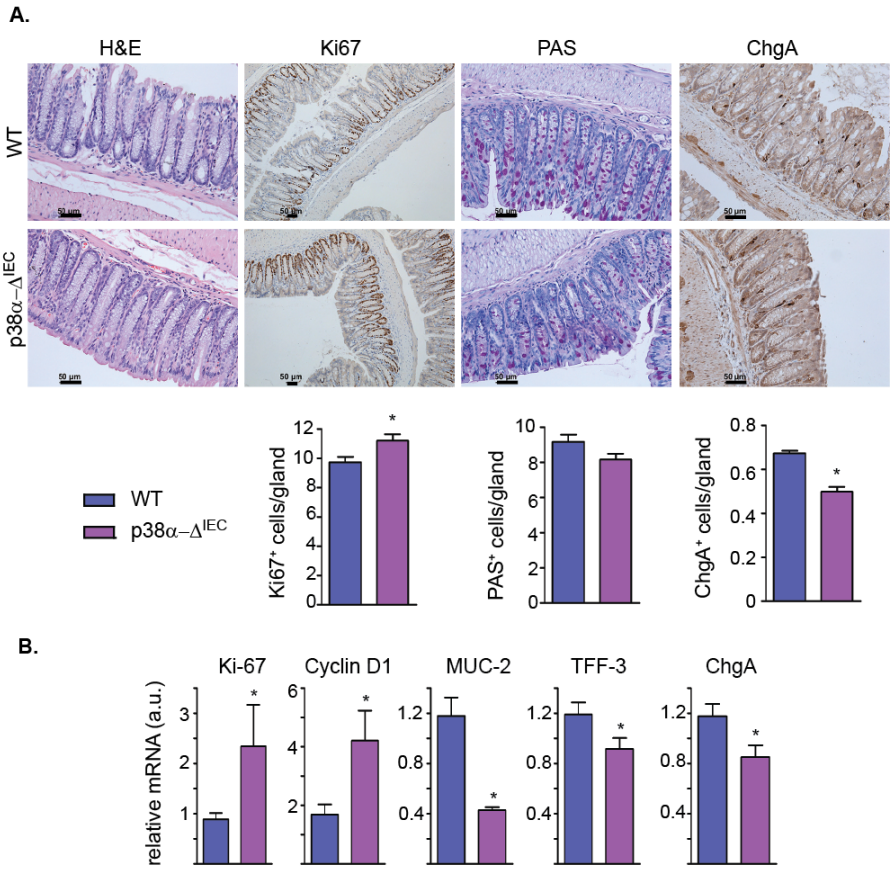


Figure 21. Downregulation of p38α in IECs affects intestinal homeostasis.
(A) Colon sections from WT and p38α-Δ^{IEC} mice were stained with H&E, Ki67 antibody for proliferation, Periodic acid-Schiff (PAS) reagent for goblet cells and Chromogranin A (ChgA) antibody for enteroendocrine cells. Quantifications were performed by counting more than 100 correctly-oriented crypts from a minimum of 5 distal colon fields. Data are means ± SEM (n = 4) *, p<0.05.
(B) Relative mRNA expression levels for the indicated genes in the distal colon of non-treated WT and p38α-Δ^{IEC} mice was determined by qRT-PCR and normalized to GAPDH mRNA. Data are means ± SEM (n = 4) *, p<0.05

Analysis of the expression of genes involved in cell proliferation showed that Ki67 and cyclin D1 mRNA levels were significantly higher in p38α-Δ^{IEC} mice, while the expression of mRNAs related to goblet cell differentiation, such as Mucin 2(MUC2) and Trefoil factor 3 (TFF3), or the enteroendocrine-specific gene ChgA was significantly lower in p38α-Δ^{IEC} mice **(Figure 21B)**. These results indicate that p38α signaling can regulate both proliferation and differentiation of IECs.

Increased intestinal permeability in p38α-Δ^{IEC} mice

We also investigated the effect of p38α downregulation on the intestinal permeability. Using FITC-dextran, we found that *in vivo* intestinal permeability was notably increased in p38α-Δ^{IEC} mice compared to WT mice **(Figure 22A)**. We then analyzed the morphology of epithelial tight junctions by electron microscopy. Tight junctions are intercellular junctional complexes that regulate the paracellular permeability and are important for integrity of the epithelial barrier. On ultrathin sections analyzed by electron microscopy, each tight junction contain a series of apparent fusions called kissing points where the intercellular space is completely obliterated (Tsukita et al., 2001). We found that epithelial tight junctions in colon from p38α-Δ^{IEC} mice were morphologically disrupted and had fewer kissing points compared to tight junctions in colon from WT mice **(Figure 22B,** arrowheads**)**. ZO-1 is a key component of tight junctions, which helps other proteins like claudins and occludin to assemble at tight junction. Expression of ZO-1 was reduced in p38α-Δ^{IEC} mice, while expression of Claudin-1 and occludin did not seem to be significantly changed in p38α-Δ^{IEC} mice compared to WT mice **(Figure 22C and 22D)**.

Taken together, the altered intestinal homeostasis and epithelial barrier function observed in p38α-Δ^{IEC} mice probably account for their enhanced susceptibility to DSS-induced colitis and epithelial damage as well as to CAC.

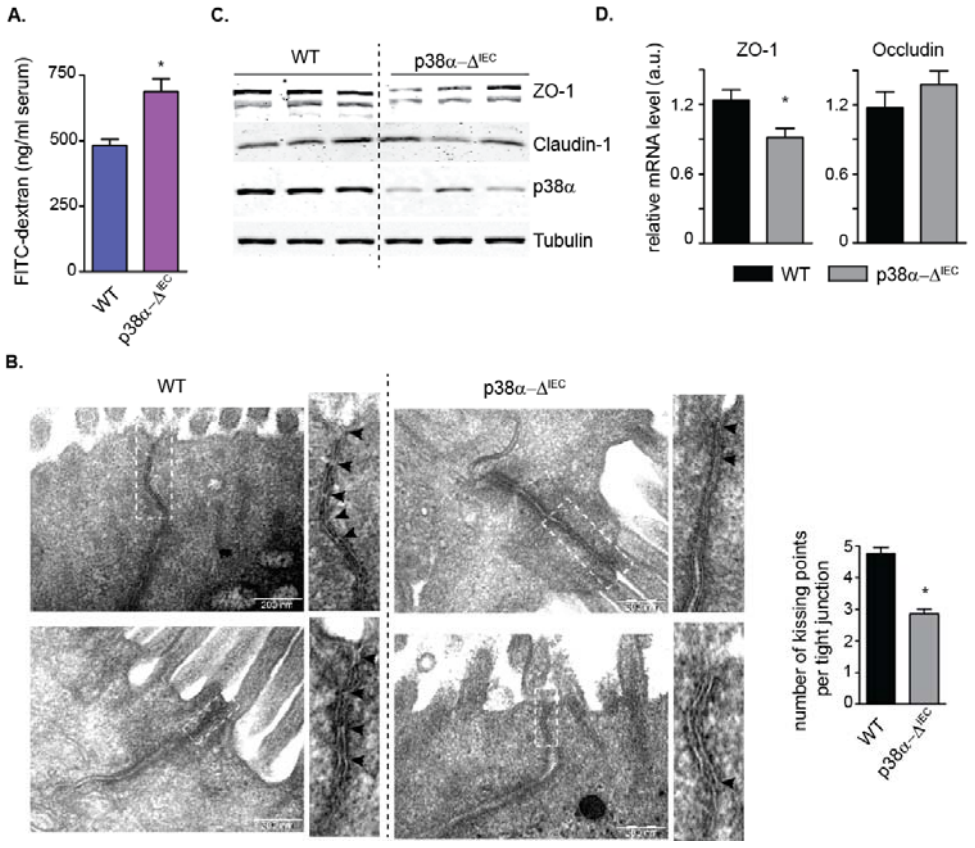


Figure 22. Downregulation of p38α in IECs affects intestinal permeability and tight junctions.

(A) Intestinal permeability was measured by determining the concentration in blood serum of FITC-dextran that was orally administered to mice. Data are means ± SEM (n = 4).

(B) Representative electron microscope images showing tight-junctions in colon epithelia from WT and p38α-Δ^{IEC} mice. At least 25 tight junctions were analyzed. Histogram shows the quantification of the number of kissing points per tight junction. Data are means ± SEM.

(C) Whole colon lysates were prepared from WT and p38α-Δ^{IEC} mice and were analyzed by western blotting with the indicated antibodies.

(D) Relative mRNA expression levels of the indicated genes in the colon of WT and p38α-Δ^{IEC} mice were determined by qRT-PCR and normalized to GAPDH mRNA. Data are means ± SEM (n = 4) *, $p<0.05$

Inducible downregulation of p38α in IECs sensitizes to AOM/DSS induced colon tumorigenesis

p38α chemical inhibitors have been reported to inhibit proliferation in some human cancer cell lines (Wagner and Nebreda, 2009). However, our genetic analyses in

mice indicate that p38α suppresses colon tumor formation. To clarify the role of p38α in colon tumorigenesis, we generated a mouse line with Villin-CreERT2 and p38α-lox alleles where p38α can be downregulated in IECs by 4-hydroxy tamoxifen (4-OHT) administration. We confirmed that treatment with 4-OHT downregulated p38α in colon but not in other tissues of p38α-lox/Villin-CreERT2 mice **(Figure 23A)**.

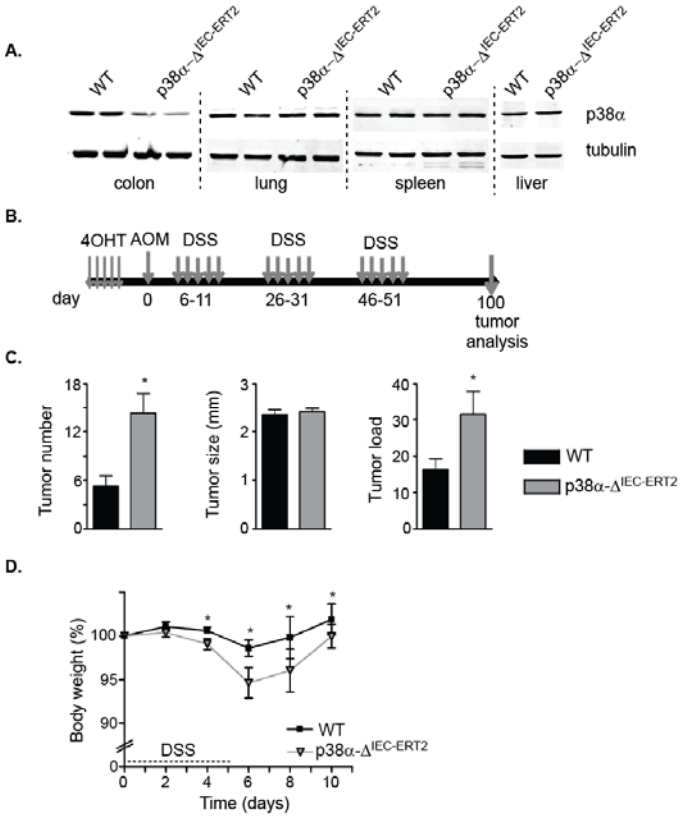


Figure 23 Inducible downregulation of p38α in IECs increases AOM/DSS-induced colorectal tumorigenesis.

(A) Colon, liver, lung and spleen lysates from 4OHT-treated mice were analyzed by Western blotting with the indicated antibodies. Lysates from 2 different mice were individually analyzed in each case (one per lane).

(B) Schematic representation of the AOM/DSS protocol used to induce colorectal tumors in p38α–$\Delta^{IEC\text{-}ERT2}$ mice.

(C) Average tumor number, tumor size and tumor load in WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice at the end of the AOM/DSS protocol.

(D) Body weight loss induced by 2% DSS administered in drinking water to WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice. Data represent means ± SEM ($n \geq 7$). *, $p<0.05$.

Next, we used these mice to confirm our previous results using constitutive Villin-Cre, by inducing p38α downregulation before the AOM/DSS treatment **(Figure 23B)**. We found that 4-OHT-treated p38α-lox/Villin-CreERT2 mice (p38α-$\Delta^{IEC\text{-}ERT2}$) developed more tumors compared to WT mice, but the average tumor size was similar **(Figure 23C)**. Moreover, p38α-$\Delta^{IEC\text{-}ERT2}$ mice lost significantly more body weight than WT mice, after 5 days of DSS treatment, as in the case of p38α-Δ^{IEC} mice **(Figure 23D)**.

Downregulation of p38α in tumor epithelial cells reduces tumor burden

Our genetic analysis showed that p38α downregulation in non-transformed IECs negatively regulates proliferation and suppresses tumor initiation. To investigate the effect of p38α in the transformed IECs, mice with p38α-lox or p38α-lox/Villin-CreERT2 alleles were subjected to the AOM/DSS protocol and 65 days after the AOM injection, the presence of colon tumors was confirmed by sacrificing a few animals. Then, p38α deletion was induced by 5 consecutive intraperitoneal injections of 4-OHT. Control mice were also injected with 4-OHT. 20 days after the last 4-OHT injection, mice were sacrificed and colon samples were harvested for tumor analysis **(Figure 24A)**.

Interestingly, a significant reduction in the number of macroscopic tumors was observed in p38α-$\Delta^{IEC\text{-}ERT2}$ mice compared to the control mice that express p38α **(Figure 24B)**. Average tumor size and tumor load were also significantly reduced upon p38α downregulation in tumor epithelial cells **(Figure 24C)**. When we analyzed tumor size distribution, we found a small difference in the number of tumors smaller than 2 mm. However, there were significantly fewer tumors of less than 4 mm in the mice where p38α was downregulated than in WT controls. Importantly, we could not detect any tumors bigger than 4 mm in p38α-$\Delta^{IEC\text{-}ERT2}$ mice **(Figure 24C)**.

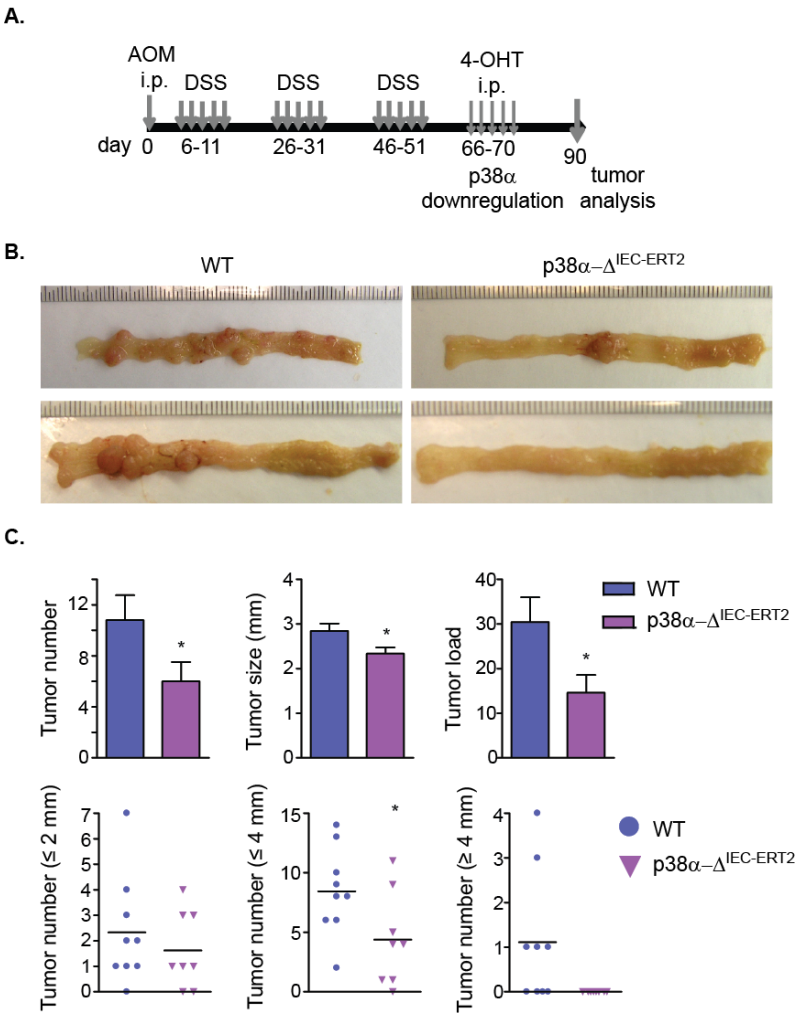


Figure 24. Downregulation of p38α in colon tumor cells reduces tumor burden

(A) Schematic representation of the protocol used to downregulate p38α in AOM/DSS-induced colon tumors.

(B) Representative images of colons from WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice showing tumors in the distal/middle region.

(C) Average tumor number, tumor size, tumor load and tumor size distribution in WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice at the end of the AOM/DSS treatment. Data represent means ± SEM (n = 8). *, $p<0.05$.

These results indicate a pro-tumorigenic role for p38α signaling in transformed colon epithelia cells, which is important for tumor maintenance.

Downregulation of p38α reduces proliferation and increases apoptosis in colon tumor cells

To determine the molecular basis for the reduced tumor burden in p38α-$\Delta^{IEC\text{-}ERT2}$ mice, we performed IHC analysis. Microscopic analysis of tumor sections stained with H&E showed smaller tumors in p38α-$\Delta^{IEC\text{-}ERT2}$ mice compared to WT mice **(Figure 25A)**. In order to check if p38α was efficiently downregulated in tumors, we performed IHC and western blot analysis. We confirmed downregulation of p38α in colon tumors and the adjacent normal epithelia by IHC as well as by western blotting in the lysates of tumors from p38α-$\Delta^{IEC\text{-}ERT2}$ mice **(Figure 25B and 25C)**.

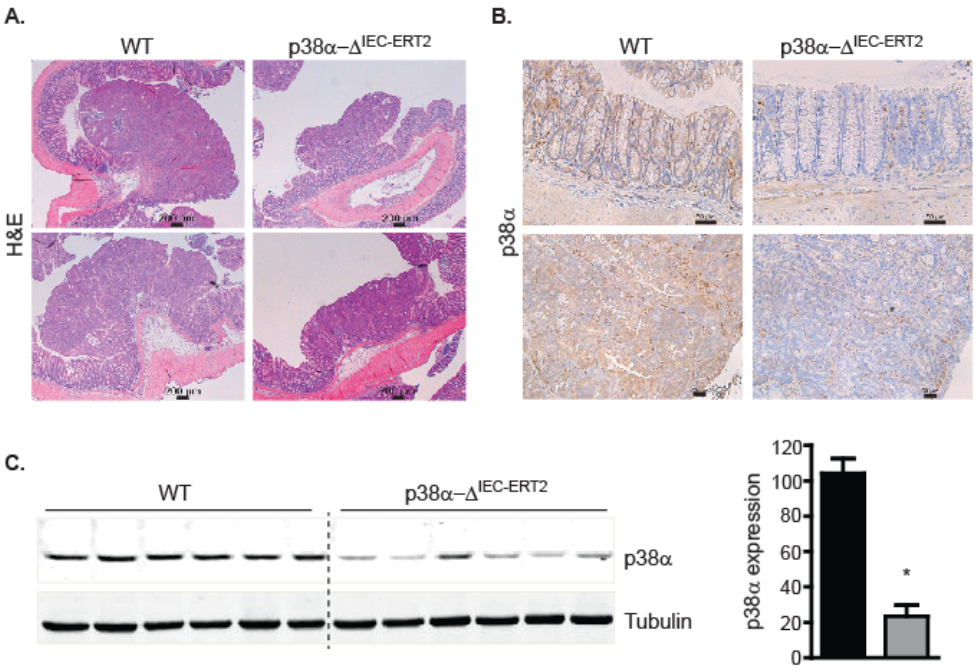


Figure 25. Downregulation of p38α in colon tumors upon 4-OHT treatment.
(A) Sections of colon tumors from WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice were stained with H&E. Bars are 200 μm.
(B) Sections of colon tumors and adjacent normal epithelia from WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice were stained with p38α antibody. Bars are 50 μm.
(C) Tumor lysates from WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice were analyzed by western blotting with p38α antibody. Typically 2 to 4 tumors were pooled from a given mouse and individually analyzed. In the right panel quantification of p38α in tumor lysates is shown. Data represent means ± SEM (n = 6). *, $p<0.05$.

Next, we analyzed the levels of proliferation and apoptosis in colon tumors from WT and p38α-$\Delta^{IEC\text{-}ERT2}$ mice, by staining with anti-Ki67 and anti-Cleaved Caspase 3 antibodies. Quantification of Ki67^{+} cells revealed significantly reduced cell proliferation in the tumors from p38α-$\Delta^{IEC\text{-}ERT2}$ mice compared to WT controls, whereas Cleaved Caspase-3^{+} apoptotic cells were more abundant in the tumors from p38α-$\Delta^{IEC\text{-}ERT2}$ mice **(Figure 26A and 26B)**. We confirmed these findings by western blotting using anti-PCNA antibody as a proliferative marker and anti-total Caspase 3 antibody as an apoptotic marker Expression of PCNA was reduced in tumors from p38α-$\Delta^{IEC\text{-}ERT2}$ mice, whereas total Caspase 3 levels were also reduced, suggesting increase in Cleaved Caspase 3 activity, in p38α-$\Delta^{IEC\text{-}ERT2}$ mice **(Figure 26C)**.

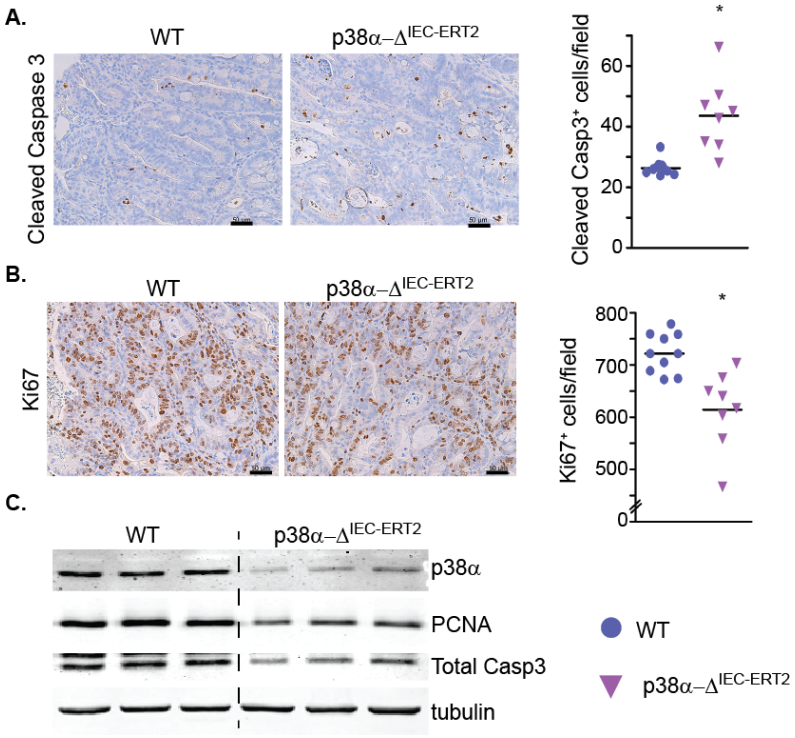


Figure 26. Downregulation of p38α in colon tumors reduces proliferation and increases apoptosis.

(A) and (B) Sections of colon tumors from WT and p38α–$\Delta^{IEC\text{-}ERT2}$ mice were stained with antibodies against cleaved Caspase 3 and Ki67. Bars are 50 μm. Average number of apoptotic cells (Cleaved caspase 3^{+}) and proliferative cells (Ki67^{+}) cells per field at the end of the AOM/DSS protocol. Data represent means ± SEM (n = 8). *, $p<0.05$.

(C) Colon lysates from WT and p38α–Δ$^{IEC-ERT2}$ mice were analyzed by western blotting with the indicated antibodies. Colon lysates from 3 different mice were individually analyzed in each case (one per lane).

We also analyzed macrophage infiltration in the tumors from both groups of mice. Quantification of F4/80^{+} macrophages revealed no significant difference in tumors from WT and p38α-Δ$^{IEC-ERT2}$ mice **(Figure 27A)**. Consistent with the importance of pro-inflammatory cytokines and chemokines such as IL-6, IL23, CXCL-1 and CXCL-2 for tumor growth (Grivennikov et al., 2009; Grivennikov et al., 2012; Jamieson et al., 2012), we also found reduced expression of these mediators in the tumors of p38α-Δ$^{IEC-ERT2}$ mice **(Figure 27B)**.

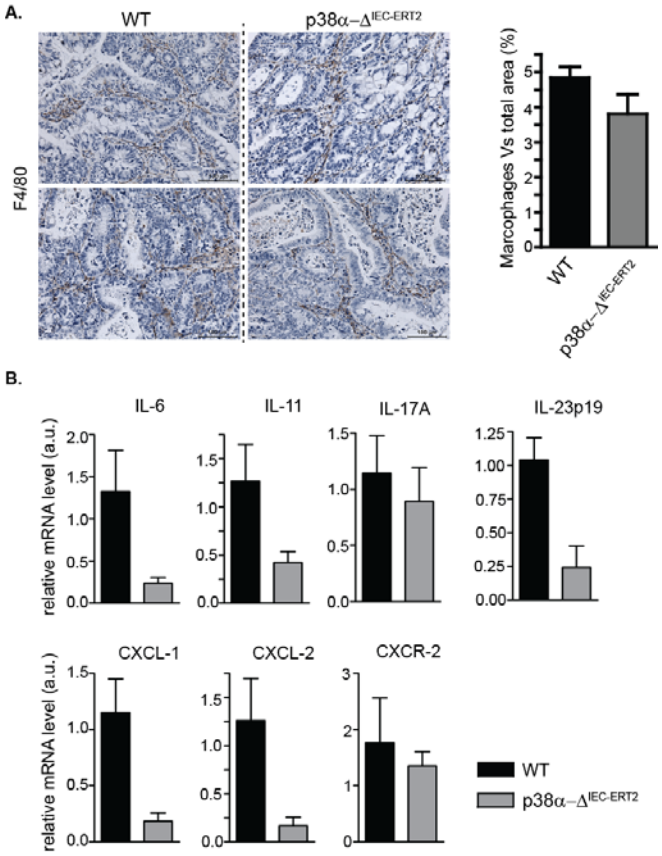


Figure 27. Inflammatory mediators in the tumors of WT and p38α–Δ$^{IEC-ERT2}$ mice.
(A) Sections of colon tumors from WT and p38α-Δ$^{IEC-ERT2}$ mice were stained with F4/80 antibody to identify macrophages.

(B) Relative mRNA expression levels for the indicated genes were determined by real-time qPCR and were normalized to GAPDH. Data are means ± SEM (n = 4).

Next we studied the effect of p38α downregulation on key signaling pathways, and found that the reduced proliferation and increased apoptosis observed in p38α-$\Delta^{IEC-ERT2}$ mice correlated with enhanced levels of phospho-JNK and reduced levels of phospho-STAT3. In contrast, the pro-survival pathways ERK1/2 and AKT did not seem to be affected by p38α downregulation in colon cancer cells **(Figure 28)**.

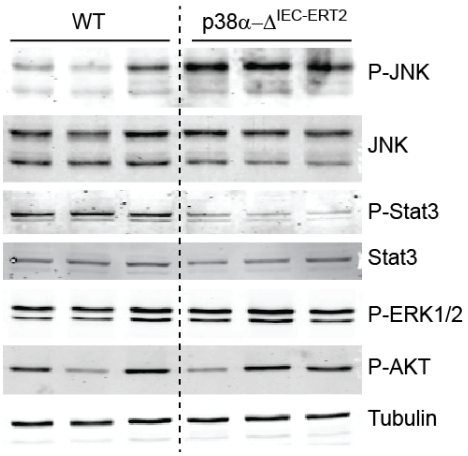


Figure 28. Signaling pathways affected by downregulation of p38α in colon tumor cells. Colon lysates from WT and p38α–$\Delta^{IEC-ERT2}$ mice were analyzed by western blotting with the indicated antibodies. Colon lysates from 3 different mice were individually analyzed in each case (one per lane).

Systemic inhibition of p38α reduces colon tumor burden

To follow up on the above findings, we investigated the effect of chemical inhibition of p38 MAPK signaling on colon tumors. We used PH797804, a novel compound that can inhibit p38α with IC50 of 26 nM and that is being used in clinical trials for Chronic Obstractive Pulmomary Disease (COPD) and Rheumatoid Arthritis (RA) (Goldstein et al., 2010). For this experiment, colon tumors were induced using the standard AOM/DSS protocol and, after confirming that tumors were formed, mice were separated in two groups. Half of the mice received PH797804 during 12 days (from day 68-80 after AOM injection), and the other half received vehicle. All the

mice were sacrificed at day 81 **(Figure 29A)**. Importantly, we found that mice treated with the p38α inhibitor had a significantly reduced colon tumor load, reflecting both less tumors and of smaller sizes compared to mice treated with vehicle **(Figure 29B)**. Histological analysis confirmed the inhibition of colon tumorigenesis by administration of PH797804, which also significantly reduced p38 MAPK signaling in the colon tumors and in the adjacent normal epithelia **(Figure 29C and 29D)**. These results confirm our genetic analysis indicating a pro-tumorigenic role for p38α signaling in colon tumor cells.

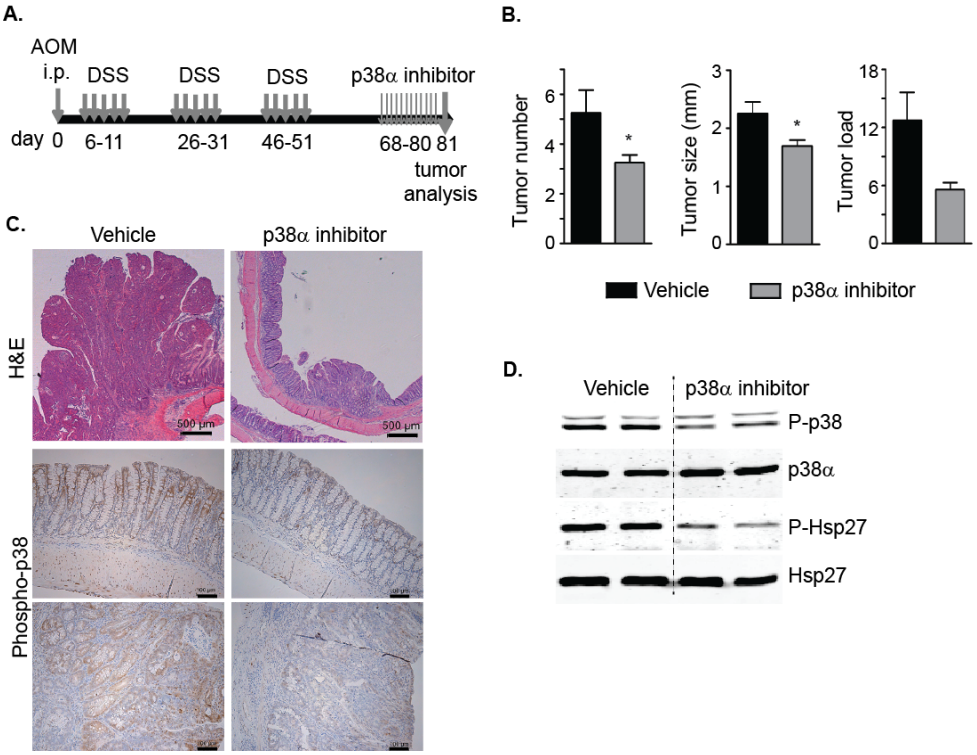


Figure 29. Chemical inhibition of p38α reduces colon tumor burden.

(A) Schematic representation of the AOM/DSS protocol used to induce colorectal tumors in C57BL/6 mice and administration of the p38α inhibitor PH797904.

(B) Average tumor number, tumor size and tumor load at the end of the AOM/DSS protocol. Data are means ± SEM (n = 7). *, $p<0.05$.

(C) Representative images of colon sections stained with H&E or the phospho-p38 MAPK antibody at the end of the AOM/DSS protocol.

(D) Colon lysates from vehicle and PH797804-treated mice were analyzed by western blotting with the indicated antibodies.

We also tested the effect of p38α inhibitors in human colon cancer cell lines. We found that incubation of Caco-2 and SW-620 cells with three different p38 MAPK inhibitors resulted in enhanced apoptosis **(Figure 30)**. Interestingly, the induction of apoptosis, detected by p85 PARP, correlated with increased levels of phospho-JNK, as observed upon p38α downregulation in the mouse colon tumors induced by AOM/DSS treatment **(Figure 30 and Figure 28)**.

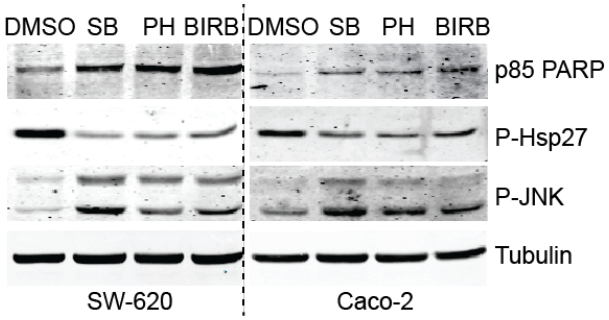


Figure 30. Inhibition of p38 MAPK induces apoptosis.
Human colon cancer cell lines SW-620 and Caco-2 were treated with the p38 MAPK inhibitors SB203580 (10 μM), PH797804 (1 μM), or Birb0796 (200 nM) for 4 days and then cell lysates were analyzed by western blotting with the indicated antibodies.

Taking together our results indicate that signaling by p38α in transformed epithelial cells contributes to colon tumorigenesis, in contrast with the tumor suppressor role of this pathway in normal colon epithelial cells.

Downregulation of p38α in myeloid cells

In order to study the role of myeloid cells (macrophages and neutrophils) in colitis and CAC, mice lacking p38α in myeloid cells (p38α-Δ^{MC}) were generated by crossing p38α (lox/lox) mice with LysM-Cre mice (Clausen et al., 1999), which express Cre recombinase under the control of the promoter for the M lysozyme gene. We confirmed the efficiency of p38α deletion in isolated peritoneal macrophages by western blotting **(Figure 31A)**. We also confirmed the deletion of exon-2 (floxed) at genomic level in isolated peritoneal macrophages. As a control B-

cells and IECs did not show any deletion of Exon-2 of p38α **(Figure 31B)**. This analysis shows the specificity of the LysM-Cre model in myeloid cells. Mice deficient in p38α in myeloid cells (p38α-Δ^{MC}) appeared to be healthy and had no obvious phenotype.

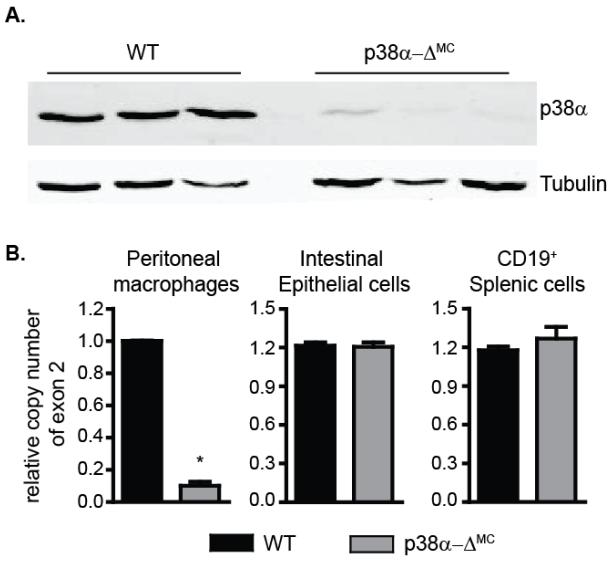


Figure 31. Analysis of p38α downregulation in p38α-Δ^{MC} mice.
(A) Western blotting of p38α in lysates prepared from isolated peritoneal macrophages from non-treated WT and p38α-Δ^{MC} mice.
(B) Genomic DNA was isolated from the indicated cell types either from WT or p38α-Δ^{MC} mice and analyzed by qPCR with primer pairs specific for exon 2 and exon 12 of the p38α. Relative copy number of exon 2 versus exon 12 is shown. Data are means ± SEM (n = 3) ∗, $p<0.05$.

Downregulation of p38α in myeloid cells does not affect DSS-induced colitis

Since p38α in myeloid cells plays a role in the production of pro-inflammatory cytokines (Kang et al., 2008; Kim et al., 2008a), we investigated the effect of inhibition of p38α in myeloid cells on DSS-induced colitis and inflammation. Mice were administered with 2% DSS for 5 days to induce acute colitis and body weight changes were recorded. We found that p38α-Δ^{MC} mice lost body weight similar to their WT control littermates. This observation suggested that p38α inhibition in myeloid cells probably has no effect on the development of DSS-induced colitis **(Figure 32A)**.

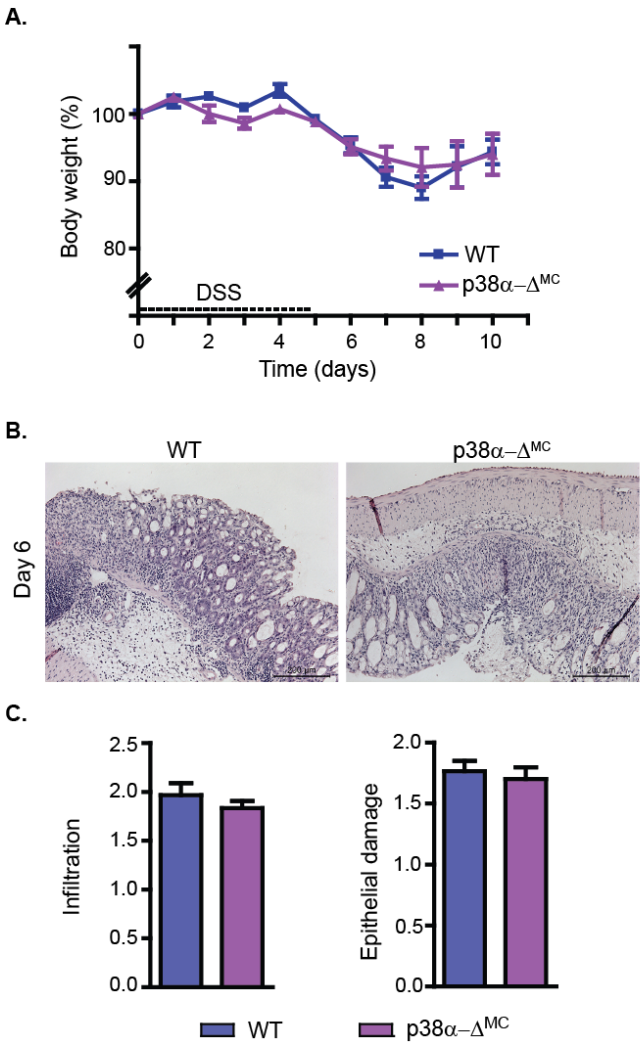


Figure 32. Downregulation of p38α in myeloid cells does not affect DSS-induced colitis.
(A) Body weight loss induced by 2% DSS in WT and p38α-Δ^{MC} mice. DSS was administered in drinking water and body weight was recorded every other day. Data are means ± SEM (n ≥ 5).
(B) Representative H&E-stained colon sections from WT and p38α-Δ^{IEC} mice that were treated with 2% DSS for 5 days and analyzed at days 6.
(C) Quantification of infiltrating immune cells and epithelial damage in H&E-stained colon sections of mice that were treated with 2% DSS for 5 days and analyzed at day 6. Data represent means ± SEM (n =4).

Next we analyzed the immune cell infiltration and epithelial damage caused by DSS administration. Mice were given 2% DSS for 5 days and then sacrificed at day 6

to perform histological analysis of colon sections **(Figure 32B)**. No significant changes in infiltration and epithelial damage were observed between colon sections from DSS-treated WT and p38α-Δ^{MC} mice **(Figure 32C)**. Since DSS is directly toxic to the epithelial layer of the colon, these results probably reflect that in both cases colon epithelial cells express p38α.

p38α in myeloid cells has been reported to be important for the inflammatory reactions in different mouse models. We therefore analyzed the mRNA expression levels of the inflammatory mediator COX-2 and pro-inflammatory cytokines such as IL-6, IL-1α and TNF-α in the colon of DSS treated mice. To our surprise, we could not detect major differences in the levels of these inflammatory mediators between DSS-treated WT and p38α-Δ^{MC} mice **(Figure 33)**.

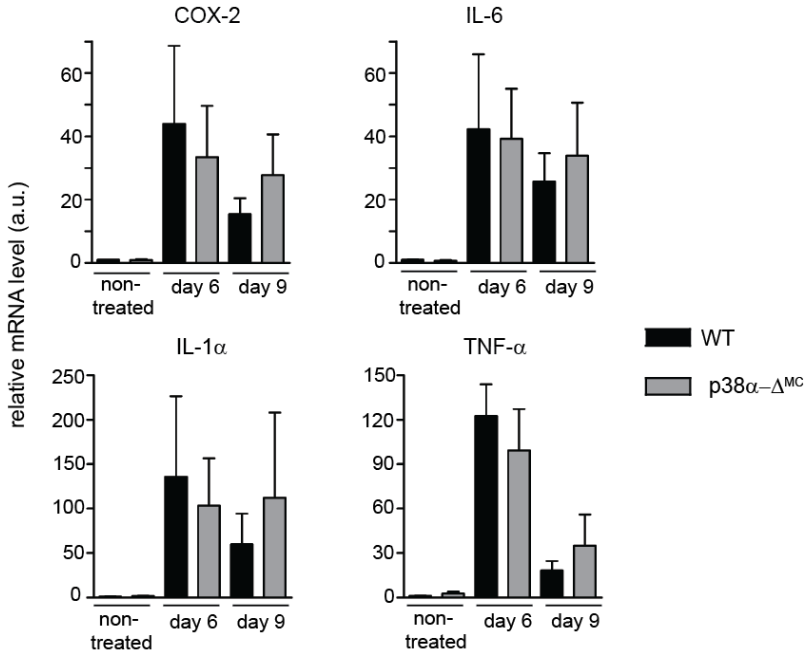


Figure 33. Inflammatory mediators in the colon of WT and p38α-Δ^{MC} mice after DSS-induced colitis.

Expression of inflammatory mediators in the distal colon of non-treated and DSS-treated WT and p38α-Δ^{MC} mice. Relative mRNA expression levels for the indicated genes were determined by real-time qPCR and were normalized to GAPDH. Expression levels in non-treated WT and p38α-Δ^{IEC} mice were the same and were given the value of 1.

These results indicate that p38α in myeloid cells does not seem to play a major role in DSS-induced acute inflammation and colitis.

Downregulation of p38α in myeloid cells does not affect CAC

In order to investigate the role of p38α in myeloid cells in the formation of AOM/DSS-induced colon tumors, we treated WT and p38α-Δ^{MC} mice with the AOM/DSS protocol. In contrast to p38α-Δ^{IEC} mice, which were more susceptible to AOM/DSS-induced tumorigenesis compared to WT mice, we did not detect differences in either number or size of colon tumors between p38α-Δ^{MC} mice and WT mice **(Figure 34A)**. Next we histologically analyzed the tumors developed in WT and p38α-Δ^{MC} mice and found tumors from both genotypes were similar in grading **(Figure 34B)**.

Consistent with the similar size of the tumors in WT and p38α-Δ^{MC} mice, we also found no significant differences in proliferation and apoptosis within tumors in both cases **(Figure 34C and 34D)**. Also there was no significant difference in the F4/80 positive macrophages within the tumors developed in WT and p38α-Δ^{MC} mice **(Figure 34E)**, indicating that p38α expression in myeloid cells might not be important for the recruitment of macrophages to colon tumors.

Taking together, these results indicate that p38α in myeloid cells does not seem to play a major role neither in AOM/DSS-induced colon tumorigenesis nor in DSS-induced acute inflammation and colitis.

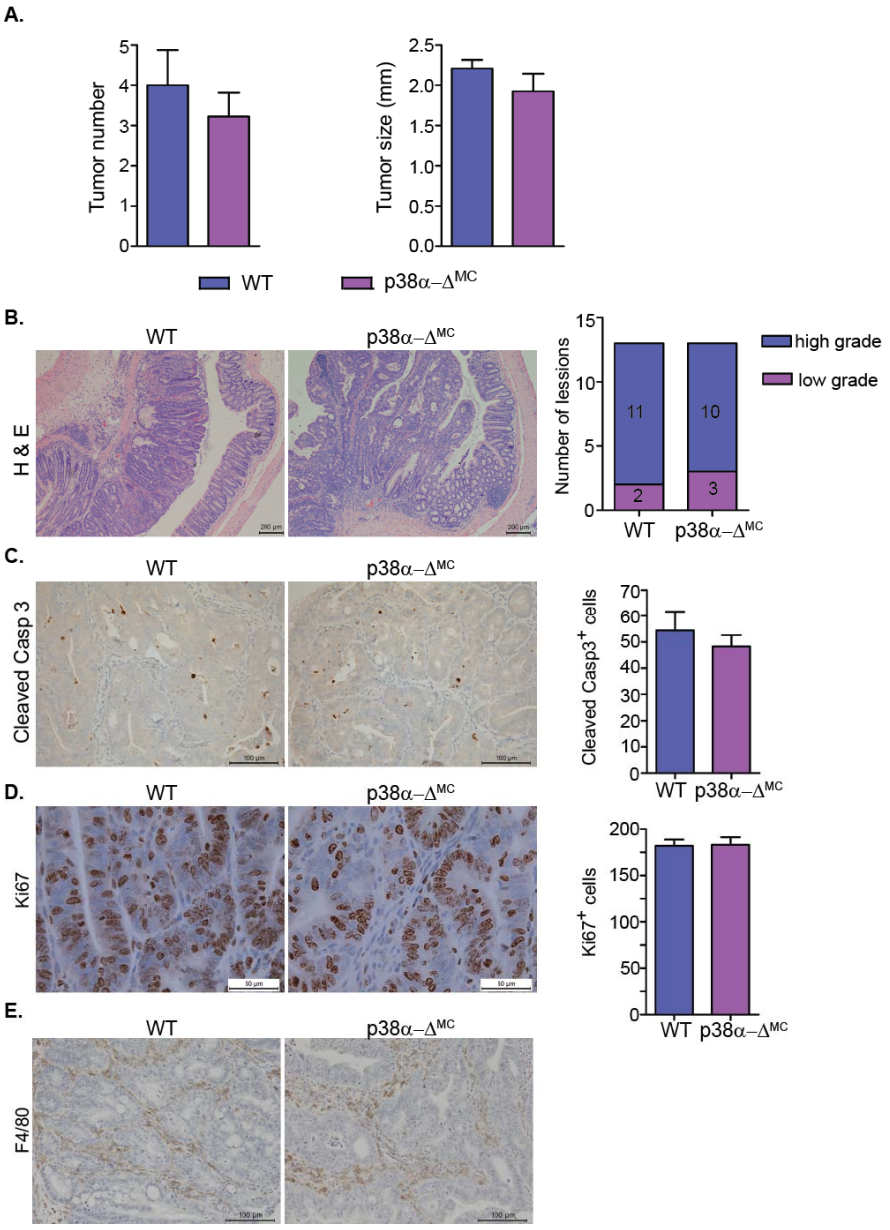


Figure 34. Downregulation of p38α in myeloid cells does not affect colorectal tumorigenesis induced by AOM/DSS.

(A) Average number and size of tumors formed in WT and p38α-Δ^{MC} mice at the end of the AOM/DSS protocol. Data represent means ± SEM (n = 9).

(B) Representative images of H&E stained colon tumors at the end of the AOM/DSS protocol. Tumors were microscopically analyzed and classified into low or high grade (n=4).

(C) & (D) Sections of colon tumors from WT and p38α–Δ^{MC} mice were stained with antibodies against cleaved caspase 3 and Ki67. Average number of apoptotic cells (Cleaved

casp 3^{+}) and proliferative cells (Ki67^{+}) per field as determined by immunohistochemistry at the end of the AOM/DSS protocol. Data represent means ± SEM (n = 4).
(E) Sections of colon tumors from WT and p38α–Δ^{MC} mice were stained with F4/80 antibody to identify macrophages.

Discussion

p38 MAPK is activated in response to many extracellular and intracellular signals and plays a vital role in cellular responses such as inflammation, proliferation, survival and differentiation. Accumulating evidence suggests the involvement of p38α in various inflammatory diseases such as asthma, rheumatoid arthritis, brain inflammation and inflammatory bowel disease and also in cancer. But little information is available about the contribution of p38α in colitis and colitis-associated cancer.

In this study, we show in a model of colon tumorigenesis that p38α performs different functions in normal and cancerous epithelial cells without having any major role in myeloid cells. On one side, epithelial p38α suppresses colitis-associated tumor initiation, whereas downregulation of p38α in myeloid cells has no effect. However, once colon tumors are formed, p38α in epithelial cells promotes tumorigenesis by facilitating proliferation and inhibiting apoptosis in the tumor cells.

1. p38α signaling in normal IECs suppresses CAC

We investigated the function of p38α in intestinal epithelial cells using the AOM/DSS model of colorectal tumorigenesis, which generates tumors that resemble human colon tumors both in distribution and at the molecular level (Neufert et al., 2007). Our results indicate that p38α downregulation in IECs enhances colitis-associated colorectal tumorigenesis. Previous reports have provided evidence for the suppression of tumor formation by p38α in mouse models of lung and liver cancer (Hui et al., 2007; Ventura et al., 2007). In both cases, enhanced tumorigenesis was associated at least in part with increased proliferation, which together with apoptosis evasion are key features in cancer development. However, we found no significant differences in proliferation and apoptosis in the AOM/DSS-induced tumors of p38α-Δ^{IEC} mice compared to WT animals. Moreover, the tumors formed by p38α deficient epithelial cells were

morphologically and histologically indistinguishable from those formed in WT mice, despite the difference in tumor incidence. These results are consistent with the observation that colon tumor size is not affected by p38α downregulation and indicate that p38α mainly suppresses tumor initiation in IEC treated with the AOM/DSS protocol.

Tumors induced by AOM/DSS are highly dependent on the inflammation and epithelial damage caused by DSS. Our results show that mice with p38α downregulation in IECs are more sensitive to DSS-induced epithelial damage, in line with a recent report (Otsuka et al., 2010). However, in contrast to Otsuka el al; we found enhanced DSS-induced inflammatory cell infiltration in p38α-Δ^{IEC} mice. The discrepancy could be due to the different mouse background, DSS dose and length of treatment. Different mouse strains show different susceptibility to DSS (Mahler et al., 1998; Stevceva et al., 1999). DSS dosage is also an important factor and we choose 2% DSS because higher DSS concentrations induced significant mortality in our mouse lines.

In addition, we show that p38α deficient IECs undergo extensive apoptosis upon DSS exposure which correlates with accumulation of the pro-apoptotic protein BAK. We have not investigated how accumulation of BAK is regulated by p38α in IECs treated with DSS. However, ionizing radiation has been reported to activate the c-Src-Rac1-p38 MAPK pathway, which attenuates BAK accumulation and cell death (Kim et al., 2008b). Epithelial apoptosis is one of mechanisms by which DSS exposure can lead to development of inflammation and colitis in various experimental mouse models. For example, deficiency of NEMO (nuclear factor kappa B (NFκB) essential modulator) in IECs results in chronic colitis due to excessive apoptosis within the epithelium (Nenci et al., 2007). Similarly, mice with specific deletion of Stat-3 in IECs are highly sensitive to DSS-induced colitis due to increased apoptosis (Bollrath et al., 2009; Pickert et al., 2009). Taking together, these

results provide evidence that excessive apoptosis in the colon epithelial cells may lead to the pathogenesis of IBD. In line with this hypothesis, there are reports showing increased apoptosis in colonic biopsies taken from IBD patients (Di Sabatino et al., 2003; Hagiwara et al., 2002; Iwamoto et al., 1996).

Enhanced epithelial damage and inflammation in p38α-Δ^{IEC} mice also correlated with increased levels of pro-inflammatory mediators COX-2 and IL-6. These results were surprising because both COX-2 and IL-6 have been described to be positively regulated by p38α (reviewed in (Wagner and Nebreda, 2009)). The increase of IL-6 and COX-2 levels in colon of DSS treated p38α-Δ^{IEC} mice is probably the result of both more infiltrating inflammatory cells and damaged epithelial cells. It has been described that in response to DEN-induced damage, p38α-deficient hepatocytes that are dying release IL-1α, which leads to induction of IL-6 and compensatory proliferation of hepatocytes resulting in hepatocellular carcinoma development (Sakurai et al., 2008). Similar to this, we show increased mRNA expression of IL-1α and IL-6 in p38α-deficient epithelial cells from DSS-treated mice. Both COX-2 and IL-6 are important for the development of colitis and CAC. COX-2 levels are elevated in patients with IBD (Singer et al., 1998). IL-6 is a critical cytokine for colorectal tumorigenesis, which regulates the survival and proliferation of IECs, and its pro-tumorigenic functions strongly relay on the activation of the transcription factor STAT3 (Becker et al., 2004; Bollrath et al., 2009; Grivennikov et al., 2009). NFκB is rapidly activated during acute inflammation, and sustained activation of this pathway can contribute to tumor development by inducing expression of inflammatory mediators and growth factors. Genetic inactivation of the NFkB pathway activator IKKβ reduces tumor burden after AOM/DSS treatment by reducing expression of cytokines and growth factors (Greten et al., 2004; Karin, 2006). Consistent with these results, the activities of both STAT3 and NFκB pathways are increased in p38α-Δ^{IEC} mice after DSS-induced colitis.

We also show that enhanced epithelial injury in p38α-Δ^{IEC} mice correlates with increased IEC proliferation during the recovery phase. This probably represents compensatory proliferation similar to what has been described in p38α-deficient hepatocytes of mice treated with the DEN carcinogen (Sakurai et al., 2008). The idea that tissue injury can contribute to cancer was hypothesized by Rudolf Virchow in 1863 (Balkwill and Mantovani, 2001; Mantovani et al., 2008). Recently, inflammation has been also proposed to play a role in injury-induced tumor development. Inflammation caused by tissue injury orchestrates wound healing and tissue regeneration, but when it cannot be properly resolved or it is propagated chronically by repetitive injury, may result in tumor promotion by uncontrolled wound healing process (Kuraishy et al., 2011). In line with this hypothesis, our results show that repetitive DSS-induced epithelial injury triggers the hyperproliferation of p38α-deficient IECs and induces colon tumor formation in the absence of AOM. These results identify p38α as a key regulator of the wound healing process, which impinges on tissue injury-induced colon tumor development.

Our results are in agreement with recent work showing that mice deficient for ASK1, a MAP3K that can activate several MAPKs including p38α, show enhanced DSS-induced epithelial injury and inflammation, and are more susceptible to AOM/DSS-induced colon tumorigenesis (Hayakawa et al., 2010). However, the effect of DSS alone on tumor formation was not evaluated in this study. DSS treatment has been reported to induce oxidative stress (Trivedi and Jena, 2012), and oxidative stress can activate ASK1-p38α signaling, which in turn negatively regulates malignant cell transformation by preventing the accumulation of oxidative stress (Dolado et al., 2007). Taking together, ASK1 may play a role upstream of p38α in the response of IECs to DSS-induced damage. Thus, oxidative stress that accumulates in absence of p38α could cooperate with increased compensatory proliferation to induce IEC transformation.

In summary, we have identified p38α as a negative regulator of CAC by controlling the apoptosis, inflammation and regeneration of the damaged colon epithelial cells.

2. p38α regulates colon epithelial cells homeostasis

The enhanced epithelial damage and inflammation induced by DSS in p38α-Δ^{IEC} mice indicate that p38α is important for maintaining homeostasis and integrity of the colon epithelia. Of particular importance is the reduced number of mucus-producing goblet cells and more proliferative cells, which we and others (Otsuka et al., 2010) have consistently observed in p38α-Δ^{IEC} mice. It has been recently shown that the p38 MAPK inhibitor SB202190 facilitates the growth of human colonic organoids in cultures, and removal of SB202190 was required to differentiate organoids into goblet and enteroendocrine cells (Sato et al., 2011). The effect of p38α may be mediated by its ability to regulate differentiation related genes like Schlafen-3 and Cdx-2 (Houde et al., 2001; Yuan et al., 2010). Mucins produced by goblet cells form a protective mucous layer, which serves as a first barrier to luminal pathogens or chemical injury. Accordingly, Muc2 knockout mice have been reported to spontaneously develop colitis and intestinal tumors (Van der Sluis et al., 2006). Despite decreased Muc2 expression in mice deficient for p38α in IEC, these mice do not develop spontaneous colitis, which may be due to insufficient reduction of Muc2 in these mice. Of note, differences in developing spontaneous colitis was very mild when compared Muc2 heterozygous mice WT mice which was more evident after DSS challenge (Van der Sluis et al., 2006), similar to p38α-Δ^{IEC} mice. These observations reflect that decreased Muc2 expression levels are likely to facilitate DSS-induced epithelial damage and tumorigenesis in the colon of p38α-Δ^{IEC} mice. It is possible that differences in genetic background might also contribute to the susceptibility of p38α-Δ^{IEC} mice to develop spontaneous colitis. For example, the severity of spontaneous inflammatory phenotype in IL-10 knockout mice has been reported to be genetic

background and husbandry conditions dependent (Buchler et al., 2012; Kuhn et al., 1993). It would be interesting to see if p38α-Δ^{IEC} mice can develop spontaneous inflammation and colitis in non-SPF animal facilities or in different genetic backgrounds.

Consistent with the expected importance of epithelial integrity in damage protection, IBD patients show altered paracellular permeability and tight junction functions (Das et al., 2012; Peeters et al., 1997; Schmitz et al., 1999; Westbrook et al., 2010). Mice lacking the tight junction protein JAM-A also show increased intestinal permeability and are more susceptible to DSS-induced colitis (Laukoetter et al., 2007). We have found that p38α-Δ^{IEC} mice show enhanced intestinal permeability, which correlates with disrupted tight junctions and reduced expression of ZO-1, an important regulator of tight junction assembly (McNeil et al., 2006). The requirement of p38α signaling to maintain epithelial homeostasis and paracellular permeability, suggest that p38α activation in IECs may provide new therapeutic opportunities for the IBD patients. Interestingly, VSL#3 probiotics can partially prevent the increased colonic permeability induced by DSS in rats, which correlates with improved expression of ZO-1 and with p38 MAPK activation (Dai et al., 2012). As probiotics mixtures can partially rescue the increased intestinal permeability, it would be interesting to use them to try to rescue some of the phenotypes associated with DSS-induced colitis such as increased apoptosis and inflammation in p38α-Δ^{IEC} mice.

In summary, our results indicate that the regulation of intestinal homeostasis by p38α may be critical to protect against colitis-induced colon epithelial damage and the initiation of colon tumorigenesis.

3. p38α signaling in transformed IECs supports colorectal tumorigenesis

Experiments using genetically modified mice, including this study, as well as cell culture models have provided good evidence for tumor suppressor functions of p38α. On the other hand, there is evidence suggesting that p38α might facilitate the proliferation and survival of established human cancer cell lines (reviewed by (Wagner and Nebreda, 2009)). However, these studies are mainly based on the use of p38 MAPK chemical inhibitors, SB203580 and SB202190, which are known to have some off target effects (Bain et al., 2007; Fabian et al., 2005). Of particular interest, SB202190 has been reported to induce autophagy in colorectal and ovarian cancer cells (Chiacchiera et al., 2009; Comes et al., 2007; Matrone et al., 2010). However, it has been described that induction of autophagic vacuoles by SB202190 is due to non-specific effect of this chemical compound rather that to the inhibition of p38 MAPK signaling (Menon et al., 2011). Nevertheless, many cell culture based studies suggest possible pro-tumorigenic effects of p38α signaling.

Here we provide genetic and pharmacological evidence that supports the implication of p38α in colon tumor development. We show that genetic inactivation of p38α in transformed IECs reduces tumor burden, which correlates with enhanced apoptosis and impaired cell proliferation. We also showed similar results using a new potent p38α inhibitor, PH797804, which is currently under phase II clinical trials for several inflammatory diseases (Goldstein et al., 2010; Hope et al., 2009).

We found that p38α downregulation in colon tumor cells impairs the phosphorylation of STAT3, which is an important regulator of colon tumor cell survival and proliferation (Bollrath et al., 2009; Grivennikov et al., 2009). Accordingly, we also found that downregulated expression of IL-6 family cytokines in the p38α depleted colon tumors. Therefore, autocrine mechanisms, such as production of IL-6, could account for the observed link between p38α

activity and STAT3 phosphorylation, although we cannot rule out that p38α might also regulate intracellular signaling pathways leading to STAT3 phosphorylation in tumor cells, as described for head and neck cancer cell lines (Riebe et al., 2011). We also found that p38α inactivation in colon tumor cells induces JNK upregulation and there is good evidence that p38α can negatively regulate JNK signaling by several mechanisms (Wagner and Nebreda, 2009). Interestingly, sustained activation of JNK has been linked to the induction of apoptosis (Dhanasekaran and Reddy, 2008; Ventura et al., 2006), and can suppress Ras-induced tumor formation (Kennedy et al., 2003). JNK can also interact with β-catenin and can negatively regulate β-catenin signaling *in vivo* and *in vitro* (Hu et al., 2008). Moreover, JNK activity is crucial for the induction of apoptosis by various stimuli in several colon cancer lines (Collett and Campbell, 2004; El Fajoui et al., 2011; Sunayama et al., 2005; Zhang et al., 2010). Taken together, it seems likely that the induction of STAT3 phosphorylation together with downregulation of JNK signaling contribute to the pro-tumorigenic role of p38α in colon cancer cells.

Of note, similar results have been described for the MAP3K ASK1 whose genetic ablation in mice enhances susceptibility to DSS-induce colitis and CAC (Hayakawa et al., 2010). In contrast, ASK1 downregulation causes growth arrest and cell death in human colon cancer lines (Kuwamura et al., 2007). Therefore, ASK1 may play a role upstream of p38α in response to DSS. Interestingly, the PRAK/MK5 kinase, which can be activated downstream of p38α signaling, has been reported to have dual functions in skin carcinogenesis: a tumor suppressor function in epithelial cells during initiation and a pro-tumorigenic role in endothelial cells during the progression stage (Sun et al., 2007; Yoshizuka et al., 2012). Our *in vivo* genetic inactivation experiments indicate that p38α signaling plays different roles in a cell autonomous manner but depending on the transformation stage of the IECs. It

remains to be determined how p38α signaling in other cell types might contribute to colorectal tumorigenesis.

During malignant transformation, cells probably experience a wide variety of stresses becoming dependent not only on the oncogenes that initiate the transformation process but also on a number of stress managing pathways that would be required for survival and/or proliferation, what has been termed non-oncogene addiction (Luo et al., 2009). Our results indicate that p38α signaling may be important for the maintenance of colon tumors by functioning as a non-oncogenic addictive pathway in colon cancer cells. Targeting this pathway may provide new therapeutic opportunities for treatment of colorectal tumors.

Taking together, our study identifies a novel mechanism by which p38α signaling may interfere with colon tumor initiation by maintaining epithelial homeostasis and regulating inflammatory responses. In addition, we show that the p38α pathway has a completely different function once the colon tumors are formed and contributes to tumor maintenance. Thus, p38α has opposite roles in non-transformed and tumorigenic epithelial cells. These results support the importance of p38α for colon tumor maintenance and its potential interest as a therapeutic target.

4. Role of p38α in myeloid cells during DSS-induced colitis and CAC

Since p38α signaling is known to regulate inflammatory responses both *in vitro* and *in vivo*, we investigated if p38α in macrophages regulates DSS-induced colitis and CAC. In contrast to the study by Otsuka et al, our results show that myeloid cell-specific inhibition of p38α signaling does not protect against DSS-induced colitis. mRNA expression analysis of pro-inflammatory mediators also failed to reveal any considerable differences in the colon of DSS-treated WT and p38α-Δ^{MC} mice. Both studies were performed on mice with different genetic backgrounds

and the time and length of the DSS treatment were also different. This may be one of reasons for the observed discrepancies as DSS has different susceptibility on different genetic background (Mahler et al., 1998; Stevceva et al., 1999). We choose 2% DSS based on our experiments with p38α-Δ^{IEC} mice, where 2% DSS was sufficient to elicit differences between WT and p38α-Δ^{IEC} mice. Similar controversial results were published on the role of p38α in myeloid cells during atherosclerosis pathogenesis, a chronic inflammatory disease and a major cause of cardiovascular mortality (Kardakaris et al., 2011; Seimon et al., 2009). Results from these studies suggest that subtle changes in p38α activity due to experimental conditions may be responsible for different outcomes. Our studies also show that p38α in myeloid cells does not affect CAC. This is consistent with the absence of differences in intestinal inflammation and injury between WT and p38α-Δ^{MC} mice during the DSS treatment.

Taken together, our results indicate that myeloid cell specific inhibition of p38α signaling does not protect against DSS-induced colitis and CAC.

5. p38α as a potential therapeutic target for colon cancer?

p38α signaling pathway has been shown to contribute to several processes, which are considered as `hallmarks of cancer´. The majority of the cell culture experiments and the experiments in mice have provided evidence that p38α acts as a tumor suppressor, mainly due to its ability to negatively regulate proliferation and to induce cell death. Noteworthy, all the *in vivo* studies to illustrate the tumor suppressor function of p38α have been performed during the initiation and early transformation process. In contrast, few reports suggest oncogenic functions of p38α. In addition to oncogenic function of p38α, it has been shown that inhibition of p38α in combination with DNA damaging anti-cancer drugs could increase the apoptotic effect (Thornton and Rincon, 2009). Moreover, increased phosphorylation of p38α has been detected in many human cancers (Wagner and

Nebreda, 2009). It is worth mentioning that tumors are often detected in patients when they are in advance stage.

In this study we show that epithelial p38α signaling can exert opposite functions depending on the transformation stage. Based on our results, it seems that p38α signaling may be one of the non-oncogenic addiction pathways which is required for the survival and proliferation of cancer cells. Thus targeting this pathway alone or in combination with chemotherapeutic agents may provide new therapeutic opportunities for the treatment of cancer. In line with this hypothesis, we show that pharmacological inhibition of p38α reduces colon tumor burden. Another study from our lab suggests that p38α inhibition can have an additive effect with cisplatin, a widely used anti-tumor drug, in a mouse model of breast cancer (Pereira, L., PhD thesis work). Thus p38α should be considered as a target for future cancer therapies. Further studies should be aimed at better defining the role of p38α in advance tumoral stages. Genetically modified mouse models with the ability to modulate p38α activity in a time and tissue dependent manner should prove useful.

Conclusions

1. p38α regulates homeostasis and maintains the barrier function in intestinal epithelial cells.

2. In response to colitis, p38α controls epithelial cell damage and inflammation.

3. p38α negatively regulates colitis-associated tumor initiation in colon epithelial cells.

4. p38α is required for the proliferation and survival of colon tumor cells.

5. p38α in myeloid cells does not seem to have a major role in colitis and colitis-associated colon cancer.

6. p38α should be considered as a potential therapeutic target in colon cancer.

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Resumen en castellano

Resumen

p38α es una proteína quinasa activada por mitógenos (MAPK), muy importante en las respuestas celulares a estrés, aunque también posee otras funciones cruciales en inflamación y homeostasis de los tejidos. La inactivación genética de p38α en células mieloides ha proporcionado evidencias de la importancia de esta ruta de señalización en la producción de citoquinas y en las respuestas inflamatorias *in vivo*. Estudios recientes mostraron *in vivo* que p38α puede suprimir la iniciación de tumores de pulmón e hígado. Sin embargo, no hay muchas evidencias de mutaciones inactivantes de p38α en tumores, lo que sugiere que las células cancerígenas podrían usar esta vía de señalización para controlar múltiples procesos celulares. En línea con esta idea, el uso de inhibidores químicos ha mostrado cómo p38α puede regular positivamente la proliferación de algunas líneas de cáncer humanas.

Dado que p38α tiene un papel coordinador de las respuestas inflamatorias, mientras que también regula negativamente la tranformación celular epitelial, hemos investigado cómo se compensan estas dos funciones en la tumorogénesis colorrectal asociada a colitis (CAC). Observamos que la depleción de p38α en las células epiteliales intestinales (IECs) de ratones aumentaba la tumorogénesis asociada a colitis sin afectar al tamaño del tumor, sugiriendo que la señalización epitelial de p38α suprime la iniciación tumoral. En consecuencia, la depleción de p38α en IECs resulta en un incremento del daño epitelial asociado a colitis e inflamación, lo que puede ser explicado por el aumento de permeabilidad paracelular y alteración de la homeostasis colónica observada en las IECs deficientes en p38α. Sin embargo, no encontramos diferencias en el daño epitelial asociado a colitis, inflamación o tumorogénesis, cuando p38α fue deplecionada específicamente en las células mieloides.

Notablemente, la depleción de p38α en las células epiteliales tumorales afecta a la proliferación e incrementa la apoptosis reduciendo la carga tumoral. Nuestros resultados indican que la señalización de p38α juega un papel dual en las IECs

durante la tumorogénesis, suprimiendo los estadíos iniciales que conducen a la transformación celular, pero contribuyendo al mantenimiento de los tumores colorrectales. Además, la inhibición farmacológica de p38α redujo la carga de tumores de colon, sugiriendo que los inhibidores de esta ruta podrían ser terapéuticamente útiles para tratar el cáncer de colon.

Introducción

El cáncer es una enfermedad compleja que surge a través de un proceso mutagénico de múltiples etapas. Durante la transformación maligna, las células normales adquieren características claves como proliferación ilimitada, autosuficiencia en señales de crecimiento, falta de respuesta a señales antiproliferativas y evasión de la apoptosis (Hanahan and Weinberg, 2011). Para adquirir estas características, las células cancerosas inducen la reprogramación y reactivación de vías de señalización celulares que en circunstancias normales están fuertemente reguladas para mantener la homeostasis de los tejidos (Luo et al., 2009).

p38α es una proteína quinasa activada por mitógenos (MAPK), esencial en las respuestas celulares al estrés, pero también en diversas funciones fisiológicas, incluidas la inflamación y homeostasis de los tejidos (Cuadrado and Nebreda, 2010; Wagner and Nebreda, 2009). Varios mediadores pro-inflamatorios, incluyendo la ciclooxigenasa-2 (COX-2) y las citoquinas IL-1, TNF-α e IL-6, pueden ser regulados por p38α (Gauthier et al., 2005; Kumar et al., 2003) mediante el control de su expresión a través de NFκB (Saccani et al., 2002). La inactivación genética de p38α en células mieloides ha proporcionado evidencia de la importancia esta quinasa en la producción de citoquinas y en las respuestas inflamatorias *in vivo*, incluyendo los modelos de sepsis inducida por LPS, lesiones de piel provocadas por SDS y UVB, y colitis mediada por DSS (Kang et al., 2008; Kim et al., 2008a; Otsuka et al., 2010). Otra de las funciones de p38α es la regulación de la homeostasis de tejido mediante el equilibrio de los procesos de

proliferación y de diferenciación celular. p38α puede controlar la diferenciación de células madre embrionarias, así como de células epiteliales intestinales y de pulmón (Cuenda and Rousseau, 2007; Oeztuerk-Winder and Ventura, 2012; Otsuka et al., 2010), mientras que regula negativamente la proliferación de muchos tipos celulares, incluyendo fibroblastos, cardiomiocitos, hepatocitos, células hematopoyéticas, y células epiteliales intestinales y de pulmón (Wagner and Nebreda, 2009). Curiosamente, los ratones deficientes en p38α son más susceptibles a la tumorogénesis de pulmón inducida por K-ras, y la supresión específica de p38α en hepatocitos también promueve cáncer de hígado inducido por DEN/Pb, lo que puede ser explicado, al menos en parte, por la inhibición de la proliferación celular por p38α (Hui et al., 2007; Ventura et al., 2007). Estos resultados indican que p38α actúa como supresor de tumores *in vivo*. Sin embargo, hay poca evidencia de mutaciones que inactiven p38α en tumores, lo que probablemente refleja la capacidad de esta vía para controlar múltiples procesos celulares, y que es utilizada por las células cancerosas para supervivencia y/o proliferación. En consonancia con esta idea, hay evidencia de que p38α a veces puede regular positivamente la proliferación de líneas tumorales humanas, como de linfoma folicular, pulmón, tiroides, mama, gliomas, cáncer de cabeza y cuello, y carcinoma hepatocelular (Wagner and Nebreda, 2009). Por otra parte, se ha demostrado que SB202190 puede inducir autofagia en células de cáncer colorrectal y de ovario (Chiacchiera et al., 2009; Comes et al., 2007; Matrone et al., 2010). Sin embargo, se ha descrito que la inducción de vacuolas autofágicos por SB202190 es debido a un efecto no específico de este compuesto químico, y no a la inhibición de la señalización de p38 MAPK (Menon et al., 2011). Sin embargo, muchos estudios basados en cultivos celulares sugieren posibles efectos pro-oncogénicos de la vía de señalización de p38α.

Los tumores colorrectales son de origen epitelial, y se desarrollan a partir de mutaciones secuenciales de diversos genes, incluyendo las vías de señalización de

Wnt, β-catenina, K-ras, p53 y factor de crecimiento transformante (TGF)-β (Fearon and Vogelstein, 1990; Ullman and Itzkowitz, 2011). Estas mutaciones pueden deberse a alteraciones genéticas o pueden ser inducidas por factores ambientales, tales como la inflamación crónica del epitelio del colon. Está bien establecido que la inflamación crónica puede conducir a un mayor riesgo de varios tipos de cáncer (Schetter et al., 2010). Por ejemplo, los pacientes con enfermedad inflamatoria intestinal (IBD), enfermedad de Crohn y con colitis ulcerosa, tienen mayor riesgo de desarrollo de cáncer colorrectal que la población sana (Ekbom et al., 1990a; Ekbom et al., 1990b; Gillen et al., 1994).

Se ha demostrado que p38α regula la homeostasis del epitelio intestinal y su inflamación (Otsuka et al., 2010). Dado que ésta se ha asociado con un mayor riesgo de tumorogénesis colorrectal, en esta tesis se ha modulado la vía de señalización de p38α en dos tipos de células, las epiteliales intestinales (IECs) y las mieloides, para dilucidar el papel de p38α en la iniciación y la progresión de la tumorigénesis colorrectal asociada con la colitis crónica *in vivo*.

Resultados

La ausencia de p38α en IECs aumenta la tumorogénesis colorrectal asociada a colitis

Para investigar el papel de p38α en la tumorogénesis colorrectal asociada a colitis (CAC), se utilizó un protocolo que combina el carcinógeno azoximetano (AOM) con sulfato de dextrano sódico (DSS), inductor de colitis (Neufert et al., 2007). Cuando aplicamos este protocolo a los ratones junto con la eliminación específica de p38α en IECs (ratones p38α-Δ^{IEC}) y los controles (WT) de camada (Figura 8), todos los ratones desarrollaron tumores de colon (**Figura 8**). El número de tumores macroscópicamente aparentes era mayor en ratones p38α-Δ^{IEC} en comparación con los animales control. Sin embargo, no existe cambio significativo en el tamaño medio de los tumores (**Figura 8**).

Un posterior análisis histológico de los tumores desarrollados tras el tratamiento AOM/DSS en ratones p38α-Δ^{IEC} y control no reveló ninguna diferencia relevante en el grado tumoral (**Figura 9**), implicando a p38α en el proceso de iniciación del tumor. En consonancia con esta idea, no hemos encontrado diferencias notables en la proliferación y la apoptosis entre los tumores procedentes de ratones p38α-Δ^{IEC} y control (**Figura 9**).

A continuación se investigó si las diferencias en la tumorogénesis entre ratones p38α-Δ^{IEC} y control se debían a diferencias en la respuesta inicial al carcinógeno AOM. Tanto el daño inducido al ADN, revelado por los niveles de γ-H2AX, así como los niveles de apoptosis, medido por la forma procesada de Caspasa 3, producidos por el AOM fueron muy similares en ambas situaciones (**Figura 11**). Estos resultados indican que la ausencia de p38α en las IECs no afecta a la respuesta inicial a AOM.

Los ratones p38α-Δ^{IEC} son más susceptibles a la colitis inducida por DSS

El protocolo de AOM/DSS depende en gran medida de la inflamación causada por ciclos repetidos de DSS (Neufert et al., 2007). Puesto que no se detectó ninguna diferencia en la primera respuesta a AOM, se investigó si las diferencias observadas en la tumorogénesis se debían a variaciones en inflamación y daño epitelial provocadas por DSS. Para probar esto, a los ratones se les administró 2% DSS durante 5 días en el agua para inducir colitis aguda. En este contexto se encontró que los ratones p38α-Δ^{IEC} perdían significativamente más peso que los ratones control (**Figura 12**). El análisis histológico reveló que los ratones p38α-Δ^{IEC} tratados con DSS tenían significativamente más daño epitelial e inflamación en la parte media-distal en comparación con los controles (**Figura 12 y 13**). La cuantificación de la infiltración de células inmunes también reveló la presencia de más células infiltradas, incluyendo más macrófagos y células B en ratones p38α-Δ^{IEC} tratados con DSS. En consonancia con el aumento de células infiltradas en ratones p38α-Δ^{IEC} tratados con DSS, también encontramos un mayor nivel de

expresión de mediadores pro-inflamatorios tales como IL-6 y la COX-2 (**Figura 14**). El análisis de ARN mensajero procedente de cada tipo de población celular mostró que el aumento de mediadores pro-inflamatorios en el colon de ratones p38α-Δ^{IEC} tratados con DSS era probablemente debido a la combinación del incremento de células infiltradas y el aumento de la expresión de estos los genes en los IECs deficiente en p38α (**Figura 14**). Sin embargo, el aumento de la expresión de IL-6 correlacionaba con el aumento de la fosforilación de STAT3 y la activación de la vía NFκB detectada por la fosforilación y degradación de IκBα, una unidad inhibidora de NFκB (**Figura 15**).

Se cree que la inflamación del colon producida por DSS es debida a la inducción de apoptosis en el epitelio intestinal, lo que conlleva la disrupción física de la barrera compuesta por la mucosa, exponiendo a las células inflamatorias de la lámina propria a bacterias y productos bacterianos provenientes del lumen intestinal (Okayasu et al., 1990). Para comprobar si el aumento de la inflamación en ratones p38α-Δ^{IEC} podría deberse a la reducción en la supervivencia de células epiteliales tras el tratamiento con DSS, se analizó la apoptosis después de 3 días de exposición a DSS, encontrando más apoptosis en ratones p38α-Δ^{IEC} con respecto a los animales control. Este fenómeno podría deberse a la acumulación de la proteína pro-apoptótica BAK (**Figura 16**).

Dada la importancia de la IL-6 en la proliferación y supervivencia de las células epiteliales intestinales (Bollrath et al., 2009; Grivennikov et al., 2009), se analizó la proliferación de células gracias a la detección de Ki67 por técnicas de inmunohistoquímica en el colon de ratones p38α-Δ^{IEC} y control tratados con DSS. A día 6, un día después de terminar la administración de DSS, en las áreas donde se presentaba una completa pérdida de criptas encontramos más células proliferativas en los ratones p38α-Δ^{IEC} que en los ratones control. Dicho incremento probablemente representaba un aumento en las poblaciones de IEC junto con células inflamatorias (**Figura 17**). En los días 9 y 13, durante la reparación y la regeneración del epitelio del colon, la proliferación detectada en las IEC fue mucho

mayor en ratones p38α-Δ^{IEC} que en ratones control (**Figura 17**). Estos datos indican que la ausencia de p38α tiene como resultado la hiperproliferación de las IECs después de la lesión inducida por DSS y colitis.

Se ha mostrado que el daño continuo en tejidos presentes en un ambiente inflamatorio puede promover la tumorogénesis (Kuraishy et al., 2011). Para comprobar esta posibilidad, nuevamente inducimos daño repitiendo el tratamiento con DSS, pero en este caso sin el agente carcinógeno AOM. Curiosamente, alrededor del 60% de los ratones p38α-Δ^{IEC} desarrollaron al menos 1 tumor, mientras que los ratones control no desarrollaron ninguno (**Figura 18**). Por otra parte, encontramos más macrófagos F4/80^{+} en el colon de animales p38α-Δ^{IEC}, lo que también correlaciona con el aumento en mediadores inflamatorios, tales como COX-2 e IL-6 (**Figura 19**). En su conjunto, estos datos sugieren que el daño continuado sobre el epitelio, junto con la inflamación inducida por DSS en ausencia de p38α, resulta en un microambiente pro-tumorogénico y en la proliferación incontrolada de las IEC, que en última instancia desemboca en la inducción de hiperplasia en el colon y la formación de tumores.

p38α regula la homeostasis y la permeabilidad celular intestinales

La alteración en las funciones de la barrera epitelial se han asociado con IBD y la inflamación intestinal en seres humanos (Westbrook et al., 2010). Debido a que los ratones p38α-Δ^{IEC} en comparación con los controles presentaron una mayor pérdida de peso corporal, mayor daño epitelial, mayor inflamación e infiltración después de la colitis aguda inducida por DSS, nos llevó a proponer la hipótesis de que los ratones p38α-Δ^{IEC} podrían tener alteradas tanto la homeostasis como la permeabilidad intestinal. De hecho, cuando se analizó la proliferación y diferenciación de las IECs, se encontró que los ratones p38α-Δ^{IEC} presentaban un incremento de células proliferativas así como de células menos diferenciadas (**Figura 21**). El análisis de la permeabilidad intestinal, utilizando FITC-dextrano, reveló que los ratones p38α-Δ^{IEC} mostraban un aumento de la permeabilidad

intestinal, lo que coincide con zónulas oclusivas alteradas y reducción de ZO-1, una proteína clave para el mantenimiento de las zónulas oclusivas (**Figura 22**). En conjunto, la alteración de la homeostasis intestinal junto con una función defectuosa de la barrera epitelial presente en los ratones p38α-Δ^{IEC} probablemente sean la causa de la mayor susceptibilidad a la colitis y al daño epitelial inducido por DSS, así como el cáncer de colon asociado a colitis aguda.

La inducción de la depleción de p38α en células epiteliales tumorales reduce la carga tumoral

El uso de inhibidores químicos de p38α para inhibir la proliferación de algunas líneas celulares de cáncer humano se ha descrito previamente (Wagner and Nebreda, 2009). Sin embargo, nuestro análisis genético indica que p38α suprime la formación de tumores de colon en las IEC no transformadas. Con el fin de aclarar el papel de p38α en la progresión de la tumorogénesis de colon, hemos generado una línea de ratones con Villin-CreERT2 y alelos p38α-lox (p38α-$\Delta^{IEC\text{-}ERT2}$) donde la eliminación de p38α en IECs puede ser controlada por la administración de 4-hidroxi-tamoxifeno (4 -OHT).

En primer lugar, ratones portadores de los alelos p38α-lox/Villin-CreERT2 fueron sometidos al protocolo de AOM / DSS. Transcurridos 65 días de la inyección de AOM, la eliminación de p38α fue inducida administrando 5 inyecciones intraperitoneales consecutivas de 4-OHT. 20 días después de la última inyección de 4-OHT, los ratones fueron sacrificados y los tumores fueron analizados (**Figura 24**). Curiosamente, se observó una reducción estadísticamente significativa en el número de tumores macroscópicos, tamaño medio del tumor y la carga tumoral total en ratones p38α-$\Delta^{IEC\text{-}ERT2}$ en comparación con los ratones control que expresan p38α (**Figura 24**). Estos resultados indican un papel pro-tumorogénico para la ruta de señalización de p38α en las células epiteliales transformadas del colon, remarcando la importancia de esta ruta en el mantenimiento del tumor.

Para determinar la base molecular responsable de la reducción en la carga tumoral en los ratones p38α-$\Delta^{IEC-ERT2}$, se realizó un estudio imunohistoquímico. El análisis del marcador de proliferación celular Ki67 reveló una reducción importante en el número de células proliferativas presente en los tumores de ratones p38α-$\Delta^{IEC-ERT2}$ en comparación con los ratones control, mientras que existía una mayor muerte celular en tumores provenientes de ratones p38α-$\Delta^{IEC-ERT2}$, detectada gracias al análisis del marcador de muerte celular, la forma procesada de la caspasa-3 (**Figura 26**). A continuación se estudió el efecto de la ausencia de p38α en otras rutas claves de señalización, encontrando que la proliferación reducida y el aumento de la apoptosis observada en ratones p38α-$\Delta^{IEC-ERT2}$ correlacionaban con niveles elevados de fosfo-JNK y reducción de los niveles de fosfo-STAT3. Sin embrago, las rutas que indican pro-supervivencia, ERK1/2 y AKT, no parecieron verse afectadas por la ausencia de p38α en las células de cáncer de colon (**Figura 28**).

La inhibición sistémica de p38α reduce la carga de tumor de colon

Siguiendo la misma dirección de nuestros resultados anteriores, planteamos la hipótesis de si la inhibición química de la ruta de señalización de p38 MAPK podría tener un efecto terapéutico en tumores de colon. Para ello utilizamos el inhibidor químico denominado PH797804, un nuevo compuesto que puede inhibir específicamente p38α con un IC_{50} de 26 nM (Goldstein et al., 2010). En este experimento, se indujeron tumores de colon mediante el protocolo AOM/DSS. Una vez confirmada la formación de tumores, los ratones fueron separados en dos grupos. La mitad de los ratones recibieron el tratamiento con PH797804 durante 12 días (desde el día 68-80 después de la inyección AOM), y la otra mitad recibió el tratamiento con vehículo. Todos los ratones fueron sacrificados en el día 81 (**Figura 29**). Es importante destacar que hemos encontrado que los ratones tratados con el inhibidor de p38α tenían una carga tumoral reducida significativa desde el punto de vista estadístico, reflejado tanto en la disminución del número de tumores como en la reducción del tamaño de los mismos, comparados con los ratones control

tratados sólo con vehículo (**Figura 29**). Estos resultados amplían nuestro análisis genético previo indicando un papel pro-tumorogénico de la ruta de señalización de p38α en las células tumorales de colon.

En conjunto, nuestros resultados indican que la ruta de señalización de p38α en células epiteliales transformadas contribuye a la tumorogénesis de colon, en contraste con el papel supresor tumoral de esta vía en el epitelio del colon normal.

La supresión de p38α en las células mieloides no afecta a la colitis inducida por DSS y CAC

Con el fin de estudiar el papel de las células mieloides (macrófagos y neutrófilos) en la colitis y el cáncer colorrectal asociado a colitis, se generaron ratones carentes de p38α en células mieloides (p38α-Δ^{MC}) mediante el cruce de ratones p38α (lox/lox) con ratones LysM -Cre (Clausen et al., 1999), que expresan la recombinasa Cre bajo el promotor del gen de la lisozima M en macrófagos y granulocitos.

A estos ratones se les administró 2% DSS durante 5 días en el agua para inducir colitis aguda y los cambios de peso corporal se registraron en días alternos. Encontramos que la pérdida de peso fue similar en ratones p38α-Δ^{MC} comparados con ratones control (**Figura 32**). El análisis histológico no reveló ningún cambio significativo en la infiltración, ni tampoco se encontraron diferencias en el daño epitelial cuantificado en secciones teñidas con H&E de ratones p38α-Δ^{MC} y control tratados con DSS (**Figura 32**). El DSS actúa principalmente sobre las células epiteliales del colon, siendo directamente tóxico para la capa epitelial del colon, induciendo inflamación y colitis. No hemos detectado ninguna diferencia significativa en el daño epitelial entre ratones p38α-Δ^{MC} y control, lo que refleja una función normal de p38α en las células epiteliales del colon en ambos casos.

Con el fin de investigar el posible papel de p38α en células mieloides en la formación de tumores de colon, se trataron ratones p38α-Δ^{MC} y control con el protocolo de tumorogénesis de colon inducido por AOM/DSS, descrito

previamente para el modelo de ratones Villin-Cre. En contraste con los resultados obtenidos en ratones p38α-Δ^{IEC}, más susceptibles a la tumorogénesis inducida por AOM/DSS, no detectamos ninguna diferencia ni en el número ni en el tamaño de los tumores de colon entre los ratones p38α-Δ^{MC} y control (**Figura 34**). El análisis histológico de los tumores que desarrollaron los ratones p38α-Δ^{MC} y control no revelaron diferencias importantes en ninguno de los parámetros analizados (grado tumoral, proliferación, apoptosis y reclutamiento de macrófagos) (**Figura 34**).

En conjunto, estos resultados indican que en células mieloides p38α no parece jugar un papel importante ni en el proceso de tumorogénesis de colon inducido por AOM/DSS, ni en la inflamación y colitis aguda inducida por DSS en nuestras condiciones experimentales (véase discusión).

Discusión

En el presente estudio mostramos que p38α desarrolla diferentes funciones en un modelo de cáncer de colon, tanto en las células epiteliales normales como en las tumorales. En cambio, su papel parece despreciable en las células mieloides.

1. La vía de señalización de p38α en IECs normales suprime CAC

Estudiamos la función de p38α en células epiteliales intestinales usando el modelo de cáncer colorectal inducido por AOM/DSS, que genera tumores que se parecen tanto a nivel molecular como por su distribución a los tumores que encontramos en humanos (Neufert et al., 2007). En línea con la función supresora de tumores previamente descrita para p38α en el cáncer de pulmón e hígado (Hui et al., 2007; Ventura et al., 2007), vemos que la disminución de los niveles de la proteína p38α en las IECs aumenta la aparición de tumores asociados a colitis. Pero no encontramos diferencias significativas en cuanto al grado, tamaño medio, proliferación o apoptosis en los tumores de los ratones p38α-Δ^{IEC} cuando los comparamos con los animales control. Estos resultados indican que la proteína

p38α esta implicada principalmente en la iniciación tumoral en las células IECs tratadas con el protocolo AOM/DSS.

Los tumores inducidos con el protocolo de AOM/DSS son altamente dependientes del daño, y la inflamación causada en el epitelio por el DSS. En línea con lo publicado recientemente (Otsuka et al., 2010), nuestros resultados muestran que los animales en los que reducimos los niveles de p38α en las células epiteliales son más sensibles al daño causado por el DSS. En cambio, nosotros también observamos un aumento de infiltración celular en los ratones p38α-Δ^{IEC}. Esta discrepancia podría deberse al diferente fondo genético de los ratones, la dosis o el tiempo de tratamiento con DSS (Mahler et al., 1998; Stevceva et al., 1999).

La muerte por apoptosis de las células epiteliales es uno de los mecanismos por el cual la exposición a DSS llevaría al desarrollo de inflamación y colitis. En este sentido, observamos que las IECs deficientes en p38α expuestas al tratamiento con DSS muestran mayores niveles de apoptosis debido a la acumulación de la proteína pro-apoptotica BAK. Esta podría ser también la razón para el aumento de la inflamación que encontramos en estos ratones. En línea con esta hipótesis, existen artículos que muestran niveles altos de apoptosis en biopsias obtenidas de pacientes con tumores de IBD (Di Sabatino et al., 2003; Hagiwara et al., 2002; Iwamoto et al., 1996).

Además, el aumento del daño producido en el epitelio y la apoptosis en los ratones p38α-Δ^{IEC}, correlaciona también con mayores niveles de los mediadores pro-inflamatorios COX-2 e IL-6. Ambos son importantes para el desarrollo de la colitis y los tumores de colon asociados a colitis. Los niveles de COX-2 se encuentran elevados en pacientes con IBD (Singer et al., 1998). La IL-6 es una citoquina crítica para el desarrollo de tumores colorrectales a través de la regulación de la proliferación y supervivencia de las IECs a través del factor de transcripción

STAT3 (Becker et al., 2004; Bollrath et al., 2009; Grivennikov et al., 2009). La proteína NFκB se activa rápidamente durante la inflamación aguda y contribuye al desarrollo del tumor a través de la inducción de la expresión de los mediadores de la infamación y factores del crecimiento (Greten et al., 2004; Karin, 2006). Tanto la vía de señalización de STAT3 como de NFkB se encuentran aumentadas en los ratones p38α-Δ^{IEC} después del tratamiento con DSS.

También vemos cómo el aumento del daño que se ha producido en el epitelio de los ratones p38α-Δ^{IEC} correlaciona con un aumento de la proliferación de las IECs durante la fase de recuperación. Probablemente sea debido a una proliferación compensatoria similar a la que se ha descrito con anterioridad en otros modelos (Sakurai et al., 2008). El daño repetido en el tejido en un ambiente con alto contenido inflamatorio puede resultar en la iniciación del proceso tumoral y promoción de los estadíos tempranos de tumorogénesis debido a los procesos de reparación descontrolados (Kuraishy et al., 2011). En línea con esta hipótesis, nuestros resultados muestran que la inducción repetitiva de daño en el epitelio por el DSS produce una hiperproliferación de las IECs deficientes en p38α, lo que es suficiente para inducir la formación del tumor en ausencia de AOM. Estos resultados identifican a la proteína p38α como un regulador clave en los procesos de reparación tisular e iniciación del proceso de tumorogénesis.

Además, nuestros resultados muestran que la proteína p38α en las IECs es importante para el correcto mantenimiento de la homeostasis y la integridad del epitelio del colon. Adquiere particular importancia la observación de la reducción del número de células globet, productoras de moco, afectando a la capa protectora de mucinas que sirve como primera barrera para los patógenos o agentes dañinos. Además, los ratones p38α-Δ^{IEC} muestran un aumento de la permeabilidad intestinal que correlaciona con el mal funcionamiento de las uniones adherentes y los niveles reducidos de la proteína ZO-1. Estas alteraciones se producen también en los

pacientes con IBD (Das et al., 2012; Peeters et al., 1997; Schmitz et al., 1999). El requerimiento de la vía de señalización de p38α para mantener la homeostasis en el epitelio y la permeabilidad paracelular, sugieren que la activación de p38α en las IECs puede proporcionar nuevas oportunidades terapéuticas para los pacientes con IBD.

2. La vía de señalización de p38α en IECs transformadas promueve el desarrollo de tumores colorrectales

Diferentes modelos animales y cultivos celulares han proporcionado evidencia del papel supresor tumoral de p38α. Pero existen estudios que sugieren que p38α podría también facilitar la proliferación y supervivencia en algunas líneas celulares humanas establecidas y en algunos modelos animales (revisado por (Wagner and Nebreda, 2009)). Aunque la mayoría de los estudios se basan en el uso de inhibidores químicos de p38 MAPK que pueden tener efectos no específicos (Bain et al., 2007; Fabian et al., 2005).

Nosotros proporcionamos evidencias genéticas y farmacológicas que muestran la implicación de p38α en el desarrollo del cáncer de colon. La inactivación genética de p38α en las IECs transformadas reduce la carga tumoral, correlacionando con un aumento de la apoptosis y proliferación deficiente. Los resultados son parecidos cuando usamos un nuevo y potente inhibidor de p38α, PH797804 (Goldstein et al., 2010; Hope et al., 2009).

Observamos que la disminución de los niveles de p38α en los tumores de colon impide la fosforilación de la proteína STAT3, un importante regulador de la proliferación y supervivencia de la célula tumoral (Bollrath et al., 2009; Grivennikov et al., 2009). También encontramos un aumento en la expresión de las citoquinas de la familia de IL-6. La producción de estas citoquinas podría establecer un mecanismo autocrino uniendo la actividad de p38α y la fosforilación de STAT3; aunque no podemos descartar que p38α pudiera regular otras rutas de

señalización que regularan a su vez STAT3 (Riebe et al., 2011). Otra alteración interesante que se produce cuando reducimos los niveles de p38α es el aumento de JNK, aunque no hemos profundizado en esta conexión en esta tesis. En conjunto, parece que la inducción de la fosforilación de la proteína STAT3 junto con la disminución de la vía de señalización de JNK contribuye al papel pro-tumorogénico de p38α en el cáncer de colon de las IECs.

Durante el proceso de transformación maligna, las células probablemente sufran una gran variedad de estrés, de manera que se vuelven dependientes no sólo de los oncogenes que inician la transformación, sino también de las vías que les son necesarias para sobrevivir. Es lo que conocemos como adicción no-oncogénica (Luo et al., 2009). Nuestros resultados indican que la vía de p38α podría ser importante para el mantenimiento de los tumores de colon, funcionando como una vía adictiva no-oncogénica en las células de cáncer de colon. De esta manera, el ataque esta vía, sola o en combinación con otros agentes quimioterapéuticos, podría proporcionar nuevas oportunidades para el tratamiento del cáncer de colon.

3. El papel de p38α en las células mieloides durante la inducción de colitis por DSS y CAC

Como se ha descrito que la vía de p38α regula las respuestas inflamatorias tanto *in vivo* como *in vitro*, estudiamos si la deficiencia de esta proteína en macrófagos tenía algún efecto beneficioso en la colitis inducida por el DSS y CAC. Nuestros resultados muestras que la inhibición específica de p38α en las células mieloides no protege de la colitis inducida por el DSS. Estos resultados no coinciden con otros publicados anteriormente (Kardakaris et al., 2011; Otsuka et al., 2010; Seimon et al., 2009), lo que indicaría que las condiciones experimentales podrían producir pequeños cambios en la actividad de p38α que conducirían a resultados diferentes. Nuestros resultados demuestran que p38α en las células mieloides no afecta a la colitis asociada a cáncer colorectal. También sugieren que la inflamación y el daño

inicial son importantes para el desarrollo de CAC, ya que no observamos diferencias entre los animales control y p38α-Δ^{MC}, ni al inicio del tratamiento con DSS, ni al final en la formación de tumores.

Aún así, ese necesitan más estudios para poder definir con claridad el papel de p38α en tumores de estadíos avanzados. Los modelos de ratón modificados genéticamente con habilidad para modular la actividad en el tiempo y en el tejido de p38α serían claves para entender el papel de esta proteína en cada uno de los compartimentos celulares y las relaciones que se establecen entre ellos.

Conclusiones

1. p38α regula la homeostasis y mantiene la función protectora de las células del epitelio intestinal.

2. En respuesta a colitis, p38α controla el daño a las células epiteliales y la inflamación.

3. p38α regula negativamente la iniciación de tumores asociados a colitis en células epiteliales del colon.

4. p38α es necesaria para la proliferación y la supervivencia de las células tumorales de colon.

5. p38α en las células mieloides no parece tener un papel importante en colitis y en cáncer de colon asociada a colitis.

6. p38 α podía ser considerada como una posible diana terapéutica en cáncer de colon.